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Conservation Genetics of Eurasian Otter in South Korea

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Conservation Genetics of Eurasian Otter in South Korea

Advised by Professor Hang Lee

By Han-Chan Park

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Department of Veterinary Medicine
The Graduate School
Seoul National University

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Han-Chan Park

Department of Veterinary Medicine

The Graduate School

Seoul National University

Abstract

The Eurasian otter in South Korea had suffered a dramatic declining in population size due to the rapid industrialization, the destruction of

otter habitat, water pollution, and poaching to obtain the fur. In recent years, the otter population has been slowly recovering as the otter habitat has been improved and the species is protected by the law. However, despite this past history of the species, there has been little population genetic study on Korean otters. Therefore, in this study, genetic analysis was conducted on 70 tissue samples of Eurasian otters using 14 microsatellite markers to identify population genetic characteristics of otter population in South Korea. The four microsatellite markers showed significant deviations from the Hardy-Weinberg equilibrium (HWE), and this might be an evidence that there was a Wahlund effect caused by the population structure. The STRUCTURE analysis based on the Bayesian analysis confirmed that the Korean otter population was divided into two subpopulation, and the BOTTLENECK analysis identified that the Korean otter population recently underwent bottleneck. The results of population genetic analysis showed that the Korean otter population has been fragmented due to the rapid decrease of population in the past and that the population fragmentation formed a population structure that restricts gene flow.

The Japanese otter (*Lutra nippon*), once inhabited most islands of Japan, is now considered as an extinct species. Although the Japanese otter is regarded as a distinct species from the Eurasian otter (*L.*

lutra), its phylogeny and taxonomic status are based on limited information on morphological and genetic data, and thus further clarification is required. Here, we assessed the phylogenetic relationship among the genus *Lutra* and taxonomic status of *L. nippon* by using the complete sequences of cytochrome *b* gene of its holotype. The present phylogenic trees supported that the genus *Lutra* specimens largely formed monophyletic group, with *L. sumatrana* as a basal to other *Lutra* species. Within *Lutra* species, *L. nippon* was distantly related with *L. lutra*. The European otter population of *L. l. lutra* were clustered together with its subspecies, *L. l. chinensis* rather than the same subspecies, Korean otter population. The discrepancy between the genetic data and traditional taxonomy justifies the necessity of reexamination of current subspecific classification system of Eurasian otters. Level of genetic divergence between the holotype of *L. nippon* and *L. lutra* was two to three-fold lower than those among the other sister species of the Lutrinae. Based on the level of divergence between the *L. nippon* and *L. lutra*, and insufficient evidence of morphological difference between them, it is suggested that designation of Japanese otter as a separate species from *L. lutra* need to be reconsidered.

The Eurasian otter, *Lutra lutra*, is well known as a threatened species in South Korea which experienced rapid population decrease by

poaching and industrialization until the 1980's. To evaluate the genetic diversity and the existing number of otters recently found in Daegu City, 81 fecal samples collected from the Gumho River and Shincheon stream were subjected to DNA extraction, sex determination, and genetic analysis using nucleic genetic markers. Individual identification and relatedness between individuals were investigated by genotypic data using twelve microsatellite loci, and sex identification was also determined based on sequence variation of the zinc finger protein gene on sex chromosomes. Our results showed that at least seven otter individuals were identified and the kinship relationships of seven individual pairs were determined. It was concluded that otters distributed widely in Daegu City have moderate levels of genetic diversity, and close monitoring of the small-sized otter population is necessary to assist successful settlement of the otters in the area.

Keywords: Eurasian otter, Phylogenetic, Population Structure, Microsatellite marker, Fecal DNA.

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General introduction

Eurasian otter (*Lutra lutra*)

Eurasian otter (*Lutra lutra*) is a semi-aquatic mammal species belonging to Lutrinae. There are 7 genus 13 species in Lutrinae (Subfamily) of Mustelidae (Family); all of these otter species are semi-aquatic, aquatic or marine, and most otters feed fish, crustaceans and shellfish. Eurasia otters eat mainly fish, but also eat waterbirds, amphibians and crustaceans. Of 13 otter species, Eurasia otter has the widest distribution area. Otters range from the Korean peninsula to Far East Russia, Siberia, Central Asia, Southeast Asia, South Asia, and Southwest Asia, to the United Kingdom, Europe and parts of North Africa. Eurasia otters are generally divided into seven subspecies, among which the distribution range of *Lutra lutra lutra* is the largest (Hung and Law, 2016). From Europe to Korea, most of the Eurasian continent's north and central regions are inhabited by *L. l. lutra*. Although China is geographically close to Korea, the Chinese otter has been considered as a separate subspecies of *L. l. chinensis*. In addition to *L. l. barang* which inhabits Southeast Asia, *L. l. nair, L. l. monticular, L. l. aurobrunnea,* and *L. l. kutab* are distributed in India and Southwest Asia.

The European otter has become a target of fur industry in the past; overharvest, poaching, intensive industrialization and water pollution have destroyed the population and habitat after the Industrial Revolution (Kimber *et al.* 2000; Almeida *et al.* 2012). However, in the latter half of the 20th century, environmental regulations were strengthened and poaching banned, thus otter habitat has been gradually restored, and the population is gradually increasing. According to current IUCN Red Data Book, Eurasia otters belong to Near Threatened species. However, considering the fact that it was one step down from the Vulnerable stage before 2004, the situation seems to be better than in the past (Junhasz *et al.* 2014).

The exact taxonomic status of the Japanese otter, i.e. whether it a subspecies of Eurasia otter, or an independent species, is still a controversial issue. Japanese otter was traditionally identified as a subspecies of *L. lutra*, *L. l. whiteleyi* based on morphological features of its skin and skull (Gray 1867, Imaizumi 1949). Conversely, Imaizumi and Yoshiyuki (1989) and Suzuki *et al.* (1996) suggested that *L. nippon* was a distinct species, based on mophological data of skulls and molecular evidence on analysis of partial cytochrome *b* gene (224 bp). However, the Japanese otter has lost its appearance since the 1980's and was officially declared extinct by the Japanese government in 2012. Even after Japanese otter disappeared in Japan, interest in this

species has increased (Yamamoto and Ando 2011). In this situation, the recent reappearance of otters on Tsushima Island provided people with a new impetus for otter conservation and research in Japan.

Eurasian otter is the only otter species in Korea. Like Europe, they have suffered rapid population declines in the past and are recovering gradually in recent years. Since 1998, a number of studies on the distribution, ecology, diet of the species in South Korea have been conducted, but the population structure and genetic diversity of the otter is not known yet (Han 1998; Cho 2006). Therefore, in this study, we investigated the phylogenetic relationships of Korean otters, Japanese otters, Chinese otters and European otters through molecular phylogenetic analysis, and population genetic analyses were conducted to evaluate the genetic characteristics of current South Korean otter population and the genetic effect of rapid population decline in the past. Finally, we conducted a ecological genetic study to identify the genetic diversity, population size and relationships of otter inhabitants in Daegu City.

Chapter 1: The taxonomic status of East Asian otter species based on molecular evidence

Introduction

The Japanese otter (*Lutra nippon*) once inhabited most of the main Japanese islands except Hokkaido (Sasaki 1995). However, due to a rapid decline in its population, it has not been sighted since the 1980's and was officially declared extinct by the Japanese government on August 28, 2012 (Kyodo News). Since the 1980's, there has been increased attention to this species (Yamamoto and Ando 2011) and it has become the subject of several morphological and genetic studies. These studies largely concluded that *L. nippon* is a distinct species separate from the Eurasian otter *Lutra lutra* (Imaizumi and Yoshiyuki 1989; Suzuki *et al.* 1996; Endo *et al.* 2000). However, the taxonomic

status of *L. nippon* still remains uncertain because the conclusion is derived from limited morphological and genetic data.

According to Wozencraft (2005), the genus *Lutra* includes three species: the Eurasian otter, *L. lutra*; the hairy-nosed otter, *L. sumatrana*; and the Japanese otter, *L. nippon*. *L. sumatrana* and *L. nippon* have limited distributions in southeast Asia and the Japanese islands, respectively. On the contrary, *L. lutra* is widely distributed across the Eurasian continent including south of the tundra line and in north Africa, and is divided into seven subspecies (Pocock 1941; Roos *et al*. 2015): *L. l. lutra* is distributed in Eurasia from England to the Korean peninsula, excluding India, southeast Asia, and southern China; *L. l. chinensis* inhabits the southern part of China and Kimmen Island of Taiwan; and the remaining five subspecies (*L. l. barang*, *L. l. nair*, *L. l. monticola*, *L. l. kutab*, and *L. l. aurobrunnea*) are distributed in the southern part of Asia. *L. nippon* was traditionally identified as a subspecies of *L. lutra*, *L. l. whiteleyi*, which is synonymous with *L. l. lutra*, based on morphological features of its skin and skull (Gray 1867; Imaizumi 1949). Conversely, Imaizumi and Yoshiyuki (1989) suggested that *L. nippon* was a distinct species, based on skulls from the Shikoku, Honshu, and Hokkaido areas, which were morphologically distinct from the skulls of *L. lutra*, including *L. l. whiteleyi*. Likewise, Suzuki *et al*. (1996)

found *L. nippon* to be a distinct species from *L. lutra* based on genetic difference of 3.6% in the partial sequences of its cytochrome *b* gene (224 bp). The length of analyzed sequence in their study was, however, too short to define a species. Furthermore, the sample size in their study was not sufficient to compare inter- and intraspecific variation among the genus *Lutra*. Therefore, Roos *et al.* (2015) concluded that the taxonomic position of *L. nippon* remains uncertain requiring further studies and cannot view it as a separate species from *L. lutra*. Regarding morphological differences, Lau *et al.* (2016a) identified that *L. lutra* from South Korea is sexually dimorphic. Therefore, sexual dimorphism in *L. nippon* is a possible confounding effect and thus interpretation should be cautioned when using morphology to evaluate their taxonomy. Moreover, Lau *et al.* (2016b) analyszed geometric morphometrics for Craniodental morphology of the Eurasian otter in South Korea, Japan, and Taiwan to identify the skull variations between the populations, and results of the analysis revealed significant difference among three population. Waku *et al.* (2016) analyzed the phylogenetic relationship among *Lutra spp.*, including *L. nippon*, using mitochondrial genome sequences (14,740 bp) and consequently divided the Japanese populations into two lineages: *L. lutra* and another *Lutra* species or subspecies. Additionally, Waku *et al.* (2016) identified two lineages of Eurasian otter (*L. lutra*) in East Asia; one lineage comprising of Chinese otter

(*L. l. chinensis*) and another comprising of Eurasian otter (*L. l. lutra*) from South Korea and Sakhalin, Russia. However, limited sampling of the Eurasian otter range in East Asia led to phylogenetic relationships among East Asian otter populations an uncertain state. Koh *et al.* (2004) concluded that partial mitochondrial DNA sequence of Korean otter was distinct from those of European otters, but authors provided limited information on relationships of Eurasian otter populations. Therefore, phylogenetic relationship of Eurasian otters at species and subspecies level in East Asia still remains unclear and the taxonomic status of *L. nippon* remains controversial.

Genetic markers based on mitochondrial DNA, such as the cytochrome *b* gene, hypervariable portion of control region (D-loop), and cytochrome c oxidase I, have been used for phylogenetic and population genetic analysis for most mammalian taxa. Specifically, the cytochrome *b* gene sequences have been used to investigate relationships among mammalian taxa at a family - subspecific level (Ledje and Arnason 1996; John and Avise 1998; Koepfli and Wayne 1998; Bradley and Baker 2001; Kurose *et al.* 2008; Koepfli *et al.* 2008a, b). Hence, the objective of this study is to determine the molecular phylogeny of *L. nippon* using the cytochrome *b* gene and to clarify its taxonomic status. Only few Japanese otter specimens have a reliable information on locality, and therefore we focused on the relationship

among the holotype of *L. nippon* (Imaizumi and Yoshiyuki 1989), *L. lutra*, and *L. sumatrana*. We also investigated the phylogenetic relationship of Eurasian otters at subspecific level.

Materials and Methods

Samples examined in this study and sequence data from GenBank are summarized in Table 1. The holotype of *L. nippon* (Imaizumi and Yoshiyuki 1989) was employed in this study. This holotype was collected in 1972 from the Nenokubi seaside of Kochi Prefecture in Japan where its skeleton and mounted skin are preserved in the National Museum of Nature and Science, Tokyo, Japan.

Table 1. Sample and DNA sequence information used in this study

Species		ID	Locality	GenBank sequence ID
Scientific name	Common name			
Lutra nippon	Japanese otter	JP1	Nenocubi seaside, Kochi, Japan	LC006975
		JP2	Hatagun, Kochi, Japan	[1]LC050126
L. l. lutra	Eurasian otter of South Korea	KO1	Busan, South Korea	KU953401
		KO2	Gurye, South Korea	KU953402
		KO3	Gangleung, South Korea	KU953403
		KO4	Yeosu, South Korea	KU953404
		KO5	South Korea	[2]FJ236015
		KO6	South Korea	[3]EF672696
	Eurasian otter of Europe	EU1	Norway	[4]AF057124
		EU2	Portugal	[5]EF689067
L. l. chinensis	Eurasian otter of China	CH1	China (its mother came from Sichuan)	[1]LC049952
L. l. spp		CH2	China	[1]LC049378
L. l. spp		CH3	China	[1]LC049377
L. sumatra	Hairy-nosed otter		Vietnam	[6]EF472347
Aonyx capensis	African clawless otter		South Africa	[4]AF057118
Aonyx cinereus	Oriental small-clawed otter		-	[4]AF057119
Lutrogale perspicillata	Smooth-coated otter		Thailand	[6]EF472348
Lontra felina	Marine otter		Chile	[4]AF057122
Lontra longicaudis	Neotropical otter		-	[4]AF057123
Lontra canadensis	North American river otter		USA	[4]AF057121
Taxidea taxus	American badger		USA	[4]AF057132

1: Waku *et al.* (2016), 2: Jang *et al.* (2009), 3: Ki *et al.* (2010), 4: Koepfli and Wayne (1988), 5: Fernandes *et al.* (2008), 6: Koepfli *et al.* (2008)

Six *L. l. lutra* specimens from the Korean peninsula were also examined to assess the possible sequence variation among *L. l. lutra* individuals. Korean otter specimens were collected from several areas in South Korea and they were obtained from a variety of sources, including individuals that were road killed, caught in a fishing net or illegal trap, or that had been rescued as cubs but subsequently died. These tissue samples had been preserved in the Conservation Genome Resource Bank for Korean Wildlife (CGRB) and Association of Korean Otter Conservation (AKOC) with proper permits from the Cultural Heritage Administration (CHA) of South Korean government because Korean otter is designated as a natural monument species by the CHA. The sequence data of complete cytochrome *b* gene of *L. l. lutra* from Europe and South Korea, *L. l. chinensis*, *L. nippon*, *L. sumatrana*, *Aonyx capensis*, *A. cinereus*, *Lontra felina*, *Lontra longicaudis*, *Lontra canadensis* (all subfamily Lutrinae), and *Taxidea taxus* (family Mustelidae) were cited from GenBank as reference data.

Total DNA from the holotype of *L. nippon* was extracted from dried costal cartilage that had been preserved in the National Museum of Nature and Science, Tokyo, Japan. The cartilage was washed in 99% ethanol after treatment with a TE buffer. The cartilage pieces were cut into 0.5 - 1.0 cm and then decalcified with EDTA (pH 8.0) at room temperature for 5 days. DNA was extracted using an Ultra Clean™

Tissue DNA Isolation Kit (MO BIO Laboratories Inc.) following the manufacturer's protocol. All procedure of DNA extraction from the holotype specimen was performed in the clean bench for preventing contamination.

Polymerase chain reaction (PCR) amplification of the cytochrome *b* gene of the holotype of *L. nippon* was performed in a 10-μL reaction volume containing the following reagents: 10X Ex *Taq* Buffer (Takara Bio Inc.), 0.5 mM of each dNTP mix (Takara Bio Inc.), 2 μM forward and reverse primers (Figure 1, Table 2), 0.5 U Ex *Taq* (Takara Bio Inc.), and 1.0 μL template DNA. Amplification was conducted for a total of 46 cycles using the 14 primers designed in the present study (Figure 1, Table 2). The conditions for the initial 10 cycles were as follows: 94°C for 30 sec, 45°C for 20 sec, and 72°C for 20 sec; and the conditions for the remaining 36 cycles were: 94°C for 30 sec, 55°C for 20 sec, and 72°C for 20 sec. Each of the partial cytochrome *b* sequences of amplicons that were amplified by combination of 14 primers was analyzed using an IBM 3130 sequencer analyzer (Applied Biosystems™), and those sequences were aligned and assembled using Geneious Pro v5.3 to obtain the complete cytochrome *b* sequence (1,140bp) from the holotype of *Lutra nippon*.

Genomic DNA of *L. l. lutra* from Korea was extracted from the tissue

using a DNA extraction kit (Blood & Tissue Kit, Qiagen™) according to the manufacturer's manual. PCR amplification of the complete cytochrome *b* gene from the Korean population of *L. l. lutra* was carried out under the following conditions: one cycle of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 40°C for 60 sec, 72°C for 90 sec, and a final cycle of 72°C for 5 min. Each 30-μL reaction volume contained 10× PCR buffer (iNtRON Biotechnology, Inc.), 0.2 mM of each dNTP mix (iNtRON Biotechnology,Inc.), 0.5 μM forward (L14724: CGA AGC TTG ATA TGA AAA ACC ATC GTT G) and reverse (H15915: AAC TGC AGT CAT CTC CGG TTT ACA AGA C) primers (Collura *et al.* 1996), 1U *i*-Star*Taq* (iNtRON Biotechnology, Inc.), and 1.5 μL template DNA (30 ng). Six complete cytochrome *b* sequences (1,140 bp) were analyzed using an ABI3730 XL sequencer analyzer (Applied Biosystems™). Sequences were aligned using Geneious Pro v5.3 (Kearse *et al.* 2012).

The pairwise genetic distance among those sequences were calculated by using PAUP4.0 based on Kimura-2-Parameter. Jmodeltest2.1.8 was used to find the best fit substitution model of sequence evolution for constructing phylogenetic trees. A maximum-likelihood (ML) tree was reconstructed using PAUP4.0 (Swofford 2001), with an application of 1,000 pseudoreplicates of this ML tree to obtain bootstrap support values. The Bayesian inference (BI) tree was obtained using MrBayes

3.2.3 (Ronquist *et al*. 2012). BI employed four simultaneous Monte Carlo Markov chains (one cold and three heated) with 1,000,000 generations and sampled every 500 generations. The first 25% of the data points were discarded as burn-in. The consensus trees from both ML and BI were illustrated using FigTree v 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

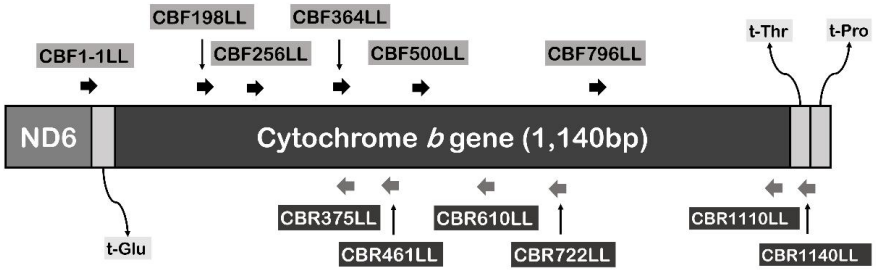


Figure 1. The location of designed primers on cytochrome *b* gene for PCR amplification of ancient DNA of Japanese otter.

Table 2. Primer list for PCR amplification of mitochondrial cytochrome *b* gene from the museum specimen of Japanese otter

Primer		
Name	Sequence	
CBF1-1LL	GTATGTCATCATTATTCCTACATGG	Forward
CBR375LL	TGCTATGGTTGCGAATAGTA	Reverse
CBF198LL	CGCACACATTTGCCGAGACG	Forward
CBR461LL	GGGATGGCTGATAGTAAGTT	Reverse
CBF256LL	GGAGCCTCCATATTCTTCAT	Forward
CBR610LL	CCTGTTTCGTGGAGAAATAGC	Reverse
CBF364LL	GCAACCATAGCAACAGCATT	Forward
CBR722LL	GGGGAGAATAGTACTAGC	Reverse
CBF500LL	GGTTCTCAGTAGACAAAGCC	Forward
CBR1110LL	GCTTGCGATTGGTATGAG	Reverse
CBF796LL	CCCCATATCAAACCTGAATGAT	Forward
CBR1140LL	GAGTCTTGGGGAGATGGGATTC	Reverse

Results

The phylogenic relationship among Lutrinae species was constructed using the ML and Bayesian methods under GTR+G substitution model, which was selected from Jmodeltest 2.1.8, based on their Akaike information criterion (AIC=8952.42) value (Figure 2). The phylogenic tree supported that the genus *Lutra* specimens largely formed monophyletic group, with *L. sumatrana* as a basal to other *Lutra* species (Figure 2, node 1). In the Chinese otter group, CH2 and CH3 were clustered with *L. l. chinensis* (CH1), so they were presumed to be the same subspecies, *L. l. chinensis*. Within *Lutra* species, the European otter population of *L. l. lutra* were clustered together with its subspecies, *L. l. chinensis* (or *L. l. spp.*) from China rather than the same subspecies, Korean otter population (Figure 2, node 2).

It is notable that a clade clustering the *L. nippon* and *L. lutra* specimens was strongly supported by both phylogenetic trees, with a bootstrap value of 100% in ML trees and a posterior probability of 100% in BI tree (Figure 2, node 3). The phylogenetic relationship showed that the holotype of *L. nippon* (JP1) and another Japanese otter (JP2) identified by Waku *et al.* (2016) were a member of the genus *Lutra* (Figure 2, node 1, 4). Furthermore, the holotype of *L. nippon* formed a monophyletic group with *L. lutra* although it was

reported that they were isolated from two other *Lutra* species, *L. lutra* and *L. sumatrana* (Imaizumi and Yoshiyuki 1989; Suzuki *et al.* 1996).

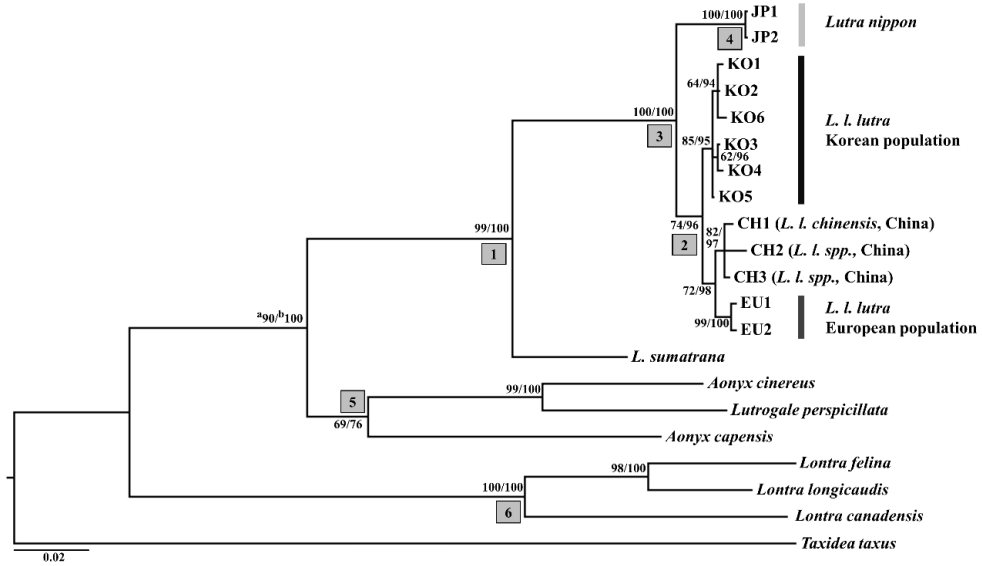


Figure 2. Phylogenetic tree reconstructed by Bayesian method using MrBayes 3.1.3, and this Bayesian tree has same topology with Maximum likelihood tree (-Ln likelihood=4427.196) reconstructed by PAUP4.0.

a: Bootstrap support values (%) obtained from 1000 pseudoreplicates of Maximum likelihood tree, b: Posterior probabilities (%) from Bayesian method.

Pairwise genetic distances of the cytochrome *b* gene among the holotype of *L. nippon*, *L. lutra*, including *L. l. lutra* and *L. l. chinensis*, and the other seven species of Lutrinae are shown in Table 3. In spite of the small sample size, our findings based on genetic distance support the distinction of *L. nippon* from *L. lutra*. The holotype of *L. nippon* had genetic distances of 2.4% - 2.8% and 2.8% - 3.3% from *L. l. lutra* and *L. l. chinensis* (or *L. l. spp.*) from China, respectively, whereas the distance between the two *Lutra* subspecies, *L. l. lutra* and *L. l. chinensis*, was only 0.8% - 1.4%. Korean and European populations of *L. l. lutra*, respectively located at the east and west extreme of Eurasian continent, had a genetic distance of 0.9% - 1.2% between them despite of long geographic distance. It is thus unlikely that *L. nippon* is part of the variation within *L. lutra*. Johns and Avise (1998) concluded that the genetic distance of the cytochrome *b* gene among mammalian sister species generally ranged from 2% to 24%. According to their index, the genetic distance between the holotype of *L. nippon* and *L. lutra* (*L. l. lutra* + *L. l. chinensis*, 2.4% - 3.3%) found in this study suggested that they differ at a marginal level. The genetic divergence between the holotype of *L. nippon* and *L. lutra* was two to three-fold smaller than those between the sister species in the Subfamily Lutrinae (6.7% - 7.2% between *L. lutra* and *L. sumatrana*, 8.1%-11.6% among *Aonyx capensis*, *A. cinereus,* and *Lutrogale perspicillata* (Figure 1, node 5), and 5.8% - 11% among *Lontra felina*,

L. longicaudis, and *L. canadensis* (Figure 1, node 6). Therefore, it is suggested that the holotype of *L. nippon* diverged from *L. lutra* at a boundary point between a subspecies and a species of the genus *Lutra*.

Table 3. Pairwise genetic distance based on cytochrome *b* gene (1,140 bp) sequence variance.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 JP1 (*Lutra nippon*)																					
2 JP2 (*L. nippon*)	.000																				
3 KO1 (*L. l. lutra*)	.026	.026																			
4 KO2 (*L. l. lutra*)	.025	.025	.001																		
5 KO3 (*L. l. lutra*)	.025	.025	.003	.002																	
6 KO4 (*L. l. lutra*)	.026	.026	.004	.003	.001																
7 KO5 (*L. l. lutra*)	.024	.024	.002	.001	.001	.002															
8 KO6 (*L. l. lutra*)	.027	.027	.003	.002	.004	.004	.003														
9 CH1 (*L. l. chinensis*)	.029	.029	.010	.009	.009	.010	.008	.011													
10 CH2 (*L. l. spp.*)	.033	.033	.013	.012	.012	.013	.012	.014	.007												
11 CH3 (*L. l. spp.*)	.028	.028	.009	.008	.008	.009	.007	.010	.003	.006											
12 EU1 (*L. l. lutra*)	.028	.028	.011	.010	.010	.011	.009	.012	.008	.012	.007										
13 EU2 (*L. l. lutra*)	.028	.028	.011	.010	.010	.011	.009	.012	.008	.012	.007	.002									
14 *L. sumatrana*	.072	.072	.069	.068	.066	.067	.067	.068	.070	.069	.067	.067	.067								
15 *Aonyx capensis*	.132	.132	.124	.123	.125	.126	.124	.123	.127	.126	.124	.126	.126	.121							
16 *Aonyx cinereus*	.129	.129	.124	.123	.126	.124	.124	.123	.126	.127	.122	.128	.128	.115	.116						
17 *Lutrogale perspicillata*	.118	.118	.109	.110	.112	.111	.111	.108	.108	.111	.107	.112	.112	.107	.113	.081					
18 *Lontra felina*	.165	.165	.158	.157	.160	.158	.158	.157	.160	.163	.161	.155	.155	.161	.167	.165	.165				
19 *Lontra longicaudis*	.170	.170	.164	.163	.165	.164	.164	.163	.165	.169	.164	.161	.161	.162	.172	.163	.162	.058			
20 *Lontra canadensis*	.165	.165	.161	.162	.162	.161	.161	.164	.169	.170	.168	.164	.164	.173	.181	.177	.177	.110	.102		
21 *Taxidea taxus*	.164	.164	.163	.162	.162	.163	.161	.162	.166	.162	.162	.165	.165	.158	.166	.185	.167	.185	.192	.195	

Discussion

The results of molecular phylogenetic analysis in this study is not in agreement with traditional subspecific taxonomic system of *L. lutra,* in which Korean otters and European otters are classified as the same subspecies (*L. l. lutra*), and otters in Southern part of China are regarded as a distinguished subspecies, *L. l. chinensis*. However, the results of this study identified haplotypes of Korean otter population as a monophyletic group distinct from the European and Chinese otter populations (Figure 2, node 2) implying that Korean otters had been branched out earlier than the divergence between *L. l. chinensis* and European otters of *L. l. lutra*. The discrepancy between the genetic data and traditional taxonomy justifies the necessity of reexamination of current subspecific classification system of Eurasian otters.

The phylogenetic relationship shown in this study was consistent with that of Waku *et al*. (2016), who used part of the 14,740 bp mitochondrial genome sequences for comparison. Waku *et al*. (2016) identified two lineages that one belonged to *L. lutra* and the other was an old Japanese lineage, and thus they were regarded as either a new *Lutra* species or a subspecies of *L. lutra*. Since complete cytochrome *b* sequences of the old Japanese lineage (JP2) in Waku *et al*. (2016) is

identical with that of the holotype of *L. nippon*, the lineage is considered to represent *L. nippon* (Figure 2. node 4).

Despite the geographic proximity of Korea to Japan than Europe, Korean otter populations are more closely related to those of Europe than to Japanese populations. A similar pattern has been reported in other mammalian species such as the Siberian flying squirrel (*Pteromys volans*), the Asiatic black bear (*Ursus thibetanus*), and the raccoon dog (*Nyctereutes procyonoides*) (Lee *et al.* 2008; Kim *et al.* 2011; Kim *et al.* 2013). The isolation and differentiation of *L. nippon* population is assumed owing to the geographic isolation of the Japanese islands from the Eurasian continent by the sea. Over time, vicariance such as the geographic isolation of *L. nippon* from *L. lutra* in the Eurasian continent may have led to its speciation. Indeed, *L. nippon* also exhibits a certain level of morphological divergence from *L. lutra*. Imaizumi and Yoshiyuki (1989) described *L. nippon* as being generally similar to *L. lutra* but with certain morphological differences, with, in the former species, a larger skull with a longer facial portion, a relatively small inner lobe of P4 (protocone), a longer tail, and a naked and larger rhinalium. Endo *et al.* (2000) also outlined some obvious differences in skull shape between *L. nippon* and the continental *L. lutra* populations using multivariate analyses. Whereas, Waku *et al.* (2016) identified two lineages of otters from Japan—*L.*

lutra and another *Lutra* species or subspecies—the latter being considered as *L. nippon* (Imaizumi and Yoshiyuki 1989). If *L. nippon* and *L. lutra* had occurred sympatrically in Japan, the evidence of genetic difference between the holotype of *L. nippon* and *L. lutra* found in our study could be explained by the existence of reproductive barriers between them. However, there is no evidence of sympatry between *L. nippon* and *L. lutra*. Both the holotype of *L. nippon* in this study and the specimen examined by Waku *et al*. (2016), which was considered to be the *L. nippon* lineage, were obtained from the Kochi Prefecture, whereas the specimen regarded as *L. lutra* in Waku *et al*. (2016) was from Jogashima, Kanagawa Prefecture. Furthermore, Waku *et al*. (2016) stated that *L. lutra* from Jogashima may have been brought there artificially. Further clarification of the taxonomic status of *L. nippon* requires the inclusion of more specimens from the Jogashima region. Although *L. nippon* has diverged from *L. lutra* at certain genetic and morphological levels, there seems insufficient evidence yet to categorize it as a distinct species.

The taxonomic uncertainty of Japanese otters is also due to the lack of phylogenetic studies of Eurasian otter subspecies. In this study, molecular genetic relationships among Korean otter (*L. l. lutra*), European otters (*L. l. lutra*), and Chinese otters (*L. l. chinensis*) are studied for the first time, but there are no studies on rest of five

subspecies (*L. l. barang*, *L. l. nair*, *L. l. monticola*, *L. l. kutab*, and *L. l. aurobrunnea*) distributed in Southeast Asia and South Asia. Phylogenetic tree and genetic distance range including remaing five subspecies of Eurasian otter will be helpful in determining the exact phylogenetic status of Japanese otters. Additionally, molecular phylogenetic studies of these Eurasian otter subspecies will reveal the evolutionary origins of the Eurasian otters, and will also contribute to otter conservation in Southeast Asia and South Asia.

Based on the level of divergence between the *L. nippon* and *L. lutra*, and limited evidence of morphological difference between them, it is, therefore, suggested that designation of Japanese otter as a separate species from *L. lutra* will be reconsidered until comprehensive and robust evidences supporting independent specific status of *L. nippon* are discovered. In addition, taxonomic classification of a regionally extirpated population as a separate species without concrete scientific evidence would not be desirable because it may preclude the potential restoration or reintroduction planning or discussion of the species into the historical range in the future.

Chapter 2: Population genetics of Eurasian otters in South Korea

Introduction

Eurasian otter (*Lutra lutra*) distributed in Eurasian continent inhabit most of rivers, lakes, coasts and reservoirs in South Korea. Eurasian otter is the top predator of fresh water ecosystem in South Korea, and serves as one of the most important indicator species of the proper functioning of the ecosystem. It also assumes the role of keystone and flagship species for biodiversity of Korean peninsula, rendering this species of high conservation value. Despite the ecological value of otters in South Korea, the population suffered dramatic decline by poaching, habitat destruction, and water pollution in 1980s. Therefore, the Eurasian otter has been protected by the law for last several decades; it was designated as a Natural Monument species (No. 330)

by the Cultural Heritage Administration in 1982, and also classified as an Endangered Species Class I by the Ministry of Environment, South Korean government. These legal protection measures and the increased public awareness on environment issues for last decades contributed improvement of the habitat and environment for otters in South Korea, resulting in a gradual increase in otter population size and distribution range in recent years (Hong 2018, Figure 9). However, there could be a possibility that the historical decrease of the otter population size and distribution range might have caused local population extinctions and fragmentation of otter habitat in the past. This might have negative effect on the genetic diversity of the population and the trace of genetic impact may be detected by molecular genetic analysis of the population. Recent historical fragmentation of the population would have formed an artificial genetic structure and reduced genetic diversity by bottleneck effect and genetic drift of the fragmented small populations. Population fragmentation and reduction of the genetic diversity could function as a negative factor in fully recovering the deteriorated population in the future. Therefore, even if the size of the otter population in South Korea is increased recently due to the improvement of otter habitat conditions, the genetic health status of the population need to be monitored and assessed by molecular genetic analysis.

There have not been extensive and comprehensive researches on the Eurasian otter, even though it has been one of the most endangered mammal species in South Korea. Nevertheless, a few researches on ecology, morphology and genetics of the species has been carried out for last decades. Most of them were ecological study including dietary study (Han 1998; Cha 2001; Nam 2004; Choi and Yoon 2012; Han 2012; Shin 2016) and some of them were on morphology (Kim 2002) and genetics (Park *et al.* 2011; Park and Cho 2017; Park *et al.* 2019). A taxonomic and phylogenetic study on East Asian populations including Korean and Japanese populations was reported recently (Park *et al.* 2019). Park *et al.* (2019) raised the need to rearrange systematic taxonomic relationships at the subspecies level between Korean otters (*Lutra lutra lutra*), Chinese otters (*L. l. chinensis*) and European otters (*L. l. lutra*). In addition, two genetic studies based on individual identification by using fecal DNA genotyping for Eurasian otters in Daegu city were carried out and the studies evaluated genetic diversity of otter populations in local areas, but nation-wide assessment of the population in whole distribution range in South Korea has not been carried out by molecular genetic method (Park *et al* 2011; Park and Cho 2017).

The microsatellite marker has been one of the most effective genetic tools for assessing genetic diversity and population structure of

endangered mammal species. The microstellites are short tandem repeat regions composed of 1~6 simple sequence unit. They have a high level of polymorphism on the number of repeating unit and show co-dominance property. Moreover, microsatellites are widely and randomly distributed across whole genome, and neutral respect to selection. These characteristics make microsatellite markers suitable for population genetic analysis. Although a new technology, the next generation sequencing, has been emerging in the field of population genetics, microsatellite markers are still being used widely in population genetics and conservation genetics researches because microsatellite loci are highly polymorphic, and each locus has multiple number of alleles. Therefore, number of alleles, allelic richness, and expected heterozygosity based on the Hardy-Weinberg equilibrium (HWE) of microsatellite loci have been used as method to estimate the levels of genetic diversity. Moreover, the difference between observed heterozygosity and expected heterozygosity under HWE on the microsatellite loci is related to whether inbreeding is in the small populations or population structures are in the large population. This HWE with Bayesian method has been used to determine the number and structures of subpopulations using population genetics softwares. In addition, population genetic analyses with the microsatellite markers may detect historical population bottlenecks by using heterozygosity excess under mutation-drift equilibrium.

Accordingly, in this study, population genetic analysis using microsatellite markers on genetic samples collected from most of otter distribution range in South Korea between 2002 and 2013 was performed for the following three purposes:

i) to assess the population structure of Eurasian otter in South Korea,

ii) to determine the cause of the population structure if there is one,

iii) and to estimate the genetic diversity of the otter population.

Materials and Methods

Sampling and DNA extraction

Seventy otter tissue (muscle or blood) samples were collected from six water systems, Han River, Nakdong River, Seomjin River, Geum River, East coast, and South coast in South Korea from 2002 to 2013 (Table 4, Figure 3). Most samples were obtained from animal carcasses that were either road-killed or caught in a fishing net or an illegal trap. Some samples were obtained from carcasses that had been rescued as orphaned cubs but subsequently died. All the tissue samples were deposited and preserved in the Conservation Genome Resource Bank for Korean Wildlife (CGRB) and Association of Korean Otter Conservation (AKOC) with proper permits from the Cultural Heritage Administration (CHA) of South Korean government because Korean otter is designated as a natural monument species by the CHA. Genomic DNA was extracted from the tissues using a DNA extraction kit (Blood and Tissue Kit, Qiagen^{TM}, USA) according to the manufacturer's manual.

Table 4. Sample information of Korean otter populations.

No.	Sample ID	Locality	Water system	Date	Sample type
1	cgrb1169	Inje, Gangwon	Han River	2004	tissue
2	cgrb1170	Inje, Gangwon	Han River	2004	tissue
3	cgrb1171	Inje, Gangwon	Han River	2004	tissue
4	cgrb2023	Chuncheon, Gangwon	Han River	2005	tissue
5	cgrb2024	Chuncheon, Gangwon	Han River	2005	tissue
6	cgrb2457	Chungju, Chungbuk	Han River	2005	tissue
7	cgrb3204	Hongcheon, Gangwon	Han River	2006	tissue
8	cgrb3913	Hongcheon, Gangwon	Han River	2006	tissue
9	akoc10045	Inje, Gangwon	Han River	2006	tissue
10	akoc10048	Inje, Gangwon	Han River	2006	tissue
11	cgrb4074	Gangwon	Han River	2007	tissue
12	cgrb4206	Suncheon, Jeonnam	South coast	2007	tissue
13	cgrb4985	Gangwon	Han River	2007	tissue
14	akoc10022	Hwacheon, Gangwon	Han River	2010	tissue
15	akoc10001	Sokcho, Gangwon	Han River	2012	tissue
16	akoc10055	Yeongwol, Gangwon	Han River	2012	tissue

Table 4. (continued.)

No.	Sample ID	Locality	Water system	Date	Sample type
17	akoc10028	Yeongwol, Gangwon	Han River	2013	tissue
18	cgrb1172	Yangyang, Gangwon	East coast	2004	tissue
19	KOA	Samcheok, Gangwon	East coast	2004	tissue
20	KOB	Gangneung, Gangwon	East coast	2004	tissue
21	cgrb2401	Gangneung, Gangwon	East coast	2005	tissue
22	cgrb2402	Gangneung, Gangwon	East coast	2005	tissue
23	akoc10051	Pohang, Gyeongbuk	East coast	2005	tissue
24	cgrb3174	Pohang, Gyeongbuk	East coast	2006	tissue
25	akoc10032	Gangneung, Gangwon	East coast	2006	tissue
26	akoc10057	Gangneung, Gangwon	East coast	2006	tissue
27	akoc10058	Gangneung, Gangwon	East coast	2006	tissue
28	cgrb6128	Uljin, Gyeongbuk	East coast	2008	tissue
29	akoc10020	Ulsan	East coast	2008	tissue
30	KOC	Yeongdong, Chungbuk	Geum River	2004	tissue
31	cgrb2833	Cheongju, Chungbuk	Geum River	2006	tissue
32	akoc10007	Cheongyang, Chungnam	Geum River	2013	tissue

Table 4. (continued.)

No.	Sample ID	Locality	Water system	Date	Sample type
33	cgrb544	Daegu	Nakdong River	2004	tissue
34	cgrb2472	Yecheon, Gyeongbuk	Nakdong River	2005	tissue
35	cgrb2473	Yecheon, Gyeongbuk	Nakdong River	2005	tissue
36	cgrb3224	Namwon, Jeonbuk	Nakdong River	2006	tissue
37	cgrb1900	Gurye, Jeonnam	Seomjin River	2005	tissue
38	cgrb1917	Gurye, Jeonnam	Seomjin River	2005	tissue
39	cgrb1943	Namwon, Jeonbuk	Seomjin River	2005	tissue
40	cgrb2866	Jinan, Jeonbuk	Seomjin River	2006	tissue
41	cgrb3713	Namwon, Jeonbuk	Seomjin River	2006	tissue
42	cgrb3986	Sunchang, Jeonbuk	Seomjin River	2007	tissue
43	akoc10027	Sunchang, Jeonbuk	Seomjin River	2013	tissue
44	cgrb90	Yeosu, Jeonnam	South coast	2002	tissue
45	cgrb128	Masan, Gyeongnam	South coast	2003	tissue
46	cgrb665	Suncheon, Jeonnam	South coast	2004	tissue
47	cgrb675	Jinhae, Gyeongnam	South coast	2004	tissue
48	cgrb801	Yeosu, Jeonnam	South coast	2004	tissue

Table 4. (continued.)

No.	Sample ID	Locality	Water system	Date	Sample type
49	cgrb802	Yeosu, Jeonnam	South coast	2004	tissue
50	cgrb1157	Jangheung, Jeonnam	South coast	2004	tissue
51	cgrb1408	Busan	South coast	2004	tissue
52	cgrb1413	Masan, Gyeongnam	South coast	2004	tissue
53	cgrb2255	Yeosu, Jeonnam	South coast	2005	tissue
54	cgrb2712	Tongyeoung, Gyeongnam	South coast	2005	tissue
55	cgrb2759	Suncheon, Jeonnam	South coast	2005	tissue
56	cgrb2990	Namhae, Gyeongnam	South coast	2006	tissue
57	cgrb3419	Suncheon, Jeonnam	South coast	2006	tissue
58	cgrb3426	Jinhae, Gyeongnam	South coast	2006	tissue
59	cgrb3585	Suncheon, Jeonnam	South coast	2006	tissue
60	cgrb3586	Suncheon, Jeonnam	South coast	2006	tissue
61	akoc10024	Busan	South coast	2006	tissue
62	cgrb4661	Geoje, Gyeongnam	South coast	2007	tissue
63	akoc10038	Haenam, Jeonnam	South coast	2007	tissue
64	cgrb6557	Sacheon, Gyeongnam	South coast	2008	tissue

Table 4. (continued.)

No.	Sample ID	Locality	Water system	Date	Sample type
65	akoc10006	Busan	South coast	2009	tissue
66	akoc10002	Haenam, Jeonnam	South coast	2010	tissue
67	akoc10033	Busan	South coast	2011	tissue
68	akoc10023	Geoje, Gyeongnam	South coast	2012	tissue
69	akoc10003	Busan	South coast	2013	tissue
70	akoc10010	Geoje, Gyeongnam	South coast	2013	tissue

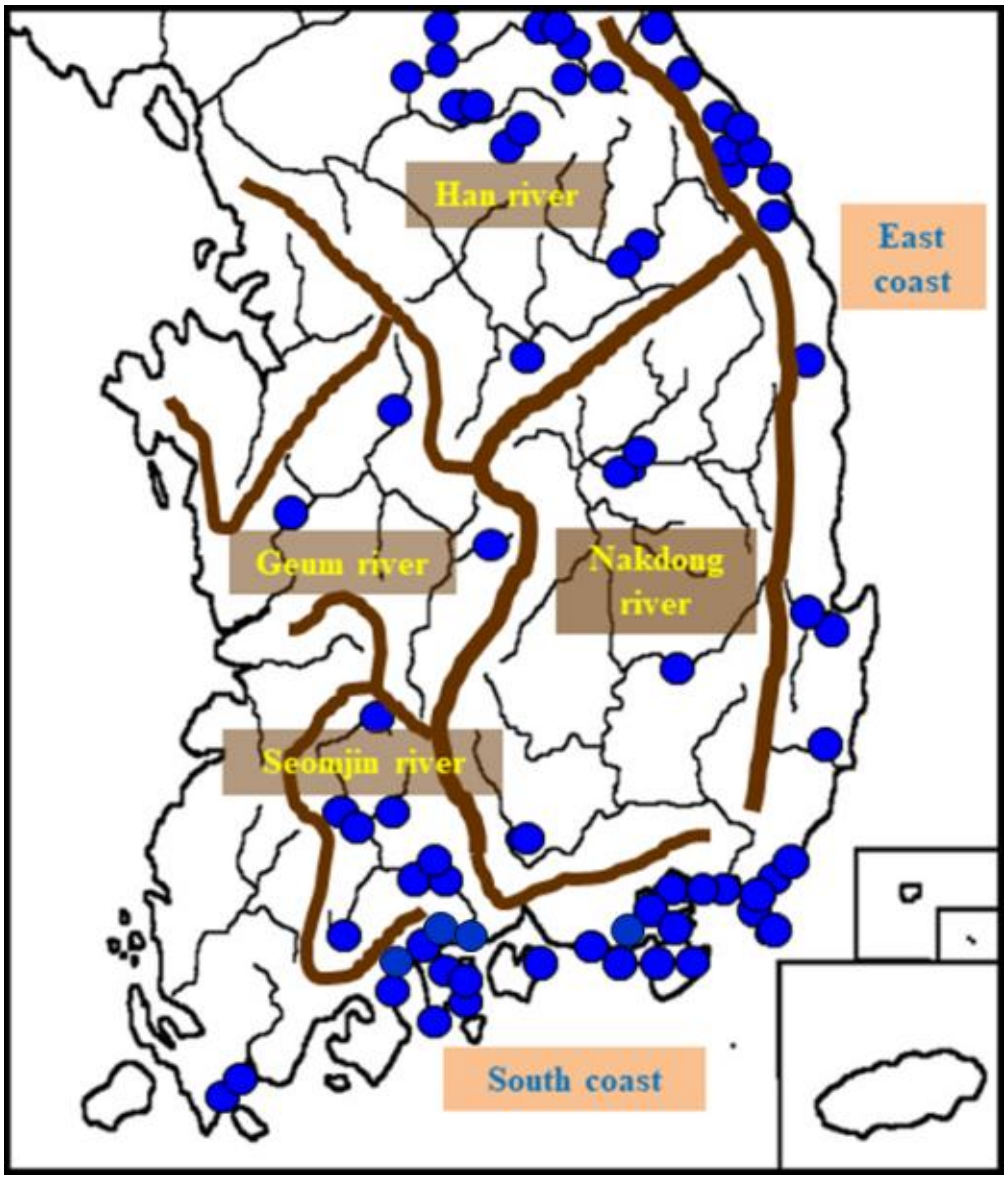


Figure 3. Sampling sites of Eurasian otter (*Lutra lutra*) in this study. Six watershed and coast areas are marked by the names of the major rivers and coasts in South Korea and the areas are divided by mountain ridges (thick brown lines).

PCR amplification and genotyping

PCR was performed in a final volume of 10 μL containing genomic DNA of otters (10 ng/μL), 0.8 mM dNTPs, 0.5 mM of each primer, and 1x PCR buffer with 1.5 mM $MgCl_2$, and 0.25 unit of i-Star Taq polymerase (Intron^{TM}). PCR products were 2 μL separated on a 1.2% agarose gel.

Fourteen microsatellite markers (Lut435, Lut453, Lut457, Lut604, Lut615, Lut701, Lut715, Lut717, Lut733, Lut782, Lut818, Lut832, Lut833, and Lut904) developed by Dallas and Piertney (1998) were used for PCR amplification for genotyping. The conditions for the initial step was: 94°C for 5 min, next 20 cycles were: 94°C for 20 sec, 60°C (-0.5 °C per cycle) for 20 sec, and 72°C for 20 sec; the conditions for the remaining 20 cycles were: 94°C for 20 sec, 50°C for 20 sec, and 72°C for 20 sec, and then final step was: 72°C for 7 min. Sizes of the alleles were determined using an ABI Prism 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) with the GENESCAN-500 ROX size standard, and GeneMapper v3.7 (Applied Biosystems, Foster City, CA, USA).

Data analysis

14 microsatellite loci were tested by MICROCHECKER 2.2.3 to detect null alleles. Linkage disequilibrium between all pair of loci were conducted with Arlequin 3.5.2.2 with 0.001 of significant level and 10,000 permutations. Genetic diversity parameters based on allelic frequency was estimated by GenAlEx ver. 6.5 using genotypic data of 14 microsatellite loci. This software estimated the number of allele, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity, and fixation index. GenAlEx software also analyzed the principle coordinates analysis by using pairwise genetic distance between individuals based on 14 microsatellite genotypic data. Mantel test for analysis of isolation by distance (IBD) was conducted by GenAlEX and Arlequin. The significance of deviation from Hardy-Weinberg equilibrium (HWE) was estimated by Genepop 4.2.

STRUCTURE 2.3.4 was used for detection of population substructure by Bayesian method. Bayesian analysis was performed by STRUCTURE based on 20 iteration per each setting K from one to six with 200,000 Markov Chain Monte Carlo (MCMC) replications after burnin 50,000 under admixture model. Likelihood data, LnP(D), was used for estimating the number of subpopulation (K) by using method of Evanno *et al.* (2005). The population diffentiation among subpopulations based on fixation index (F_{ST}) was calculated by using FSTAT 2.9.3 with 300 permutation for exact test.

The recent population bottleneck was confirmed under three microsatellite mutation models, infinite allele model (IAM), stepwise mutation model (SMM), and two phase model (TPM) which was combined with 30% of IAM and 70% of SMM by using BOTTLENECK 1.2.02. Sign test, standardized difference test, and Wilcoxon sign rank test were perfomed as statistical analsys for detecting population bottleneck, and Mode-shift analysis based on allele frequency data was also conducted. Relatively older declining of effective population size was detected based on Garza-Williamson index by AGARst ver 3.3.

Table 5. Microsatellite marker information and the results of null allele test and linkage disequlibrium analysis.

Locus	Sequence (5′ -> 3′)	Fluorescent dye	Null allele		Linkage Disequilibrium (P-value<0.001)
			Presence	Brookfield frequency	
Lut435	F - TGAAGCCCAGCTTGGTACTTC	6-FAM	No	0.0046	Ns
	R - ACAGACAGTATCCAAGGGACCTG				
Lut453	F - AGTGCTTTGTACTTGGTAATGG	6-FAM	Yes	0.0886	Ns
	R - AGACTGAAAGCTCTGTGAGGTC				
Lut457	F - CAGGTTTATGGCTTTATGGCTTTC	HEX	No	0	Ns
	R - CAGGGTTTGATTTCTGGTGAGG				
Lut604	F - TATGATCCTGGTAGATTAACTTTGTG	6-FAM	No	0.0032	Ns
	R - TTTCAACAATTCATGCTGGAAC				
Lut615	F - TGCAAAATTAGGCATTTCATTCC	HEX	No	0.0067	Ns
	R - ATTCTCTTTTGCCCTTTGCTTC				
Lut701	F - GGAAACTGTTAAAGGAGCTCACC	6-FAM	No	0.0331	Ns
	R - CAGTGTTCATAAGGATGCTCCTAC				
Lut715	F - TTCACAATAGCCAAGATATGGAC	6-FAM	Yes	0.103	Ns
	R - TGGCATAATATCCTTTCTCATGG				

Table 5. (continued.)

Locus	Sequence (5′ -> 3′)	Fluorescent dye	Null allele		Linkage Disequilibrium (P-value<0.001)
			Presence	Brookfield frequency	
Lut717	F - TGTTGCCTTCAGAGTCCTGTG	6-FAM	No	0.0291	Ns
	R - GTCAGGCATTGTAACATATTCTCAG				
Lut733	F - GATCTCATTTTAAATGTTCTTACCAC	6-FAM	Yes	0.1009	Ns
	R - TGGTTCTCTTGCAGGATCTG				
Lut782	F - GAGATATCACTAAGCAATACACGATG	6-FAM	No	0.0476	p<0.001
	R - ACAAAGACTGAGCAAAACAAGC				with Lut902
Lut818	F - AAGGATGTGAAACAGCATTG	6-FAM	Yes	0.1204	Ns
	R - CCATTTTATACACATAAATCGGAT				
Lut832	F - TGATACTTTCTACCCAGGTGTC	6-FAM	No	0.0013	Ns
	R - TCCTTAGCATTATCTTATTTACCAC				
Lut833	F - CAAATATCCTTTGGACAGTCAG	6-FAM	No	0.0378	Ns
	R - GAAGTTATCTAATTTGGCAGTGG				
Lut902	F - CAGGAGTGAATGTAAAGAGTTGG	6-FAM	No	0.0115	p<0.001
	R - CTTCACACCATTTGCAGACC				with Lut782

Ns - Not significant

Results

The genotypes of 14 microsatellite loci were identified from DNA samples of 70 Eurasian otter individuals. The observed heterozygosity were calculated from these genotypic data, and then expected heterozygosity also were determined from allelic frequency data based on HWE by using GenAlEx. In the results of comparing between observed and expected heterozygosity, four microsatellite loci, Lut453, Lut715, Lut733, and Lut818 were deviated from HWE (Table 5), and also these four loci were identified as possibly having null alleles based on MICROCHECKER analysis (Table 5). Since the null allele was identified in the four markers, additional analysis was conducted to determine the cause. To see if the null allele detection was due to the Wahlund effect by the population substructure, the null allele test was performed again for each of the north and south population separately. The result showed that the null allele was not detected in the north population, and null allele was confirmed in only one locus in the south population (Table 7). The result suggest that the detection of null allele might be caused by Wahlund effect due to otter population substructure in South Korea.

The range of allele number was between 4 and 17 for the 14 microsatellite loci (Table 6). Based on the analysis of allele frequency

data for the 14 loci, linkage disequilibrium between Lut782 and Lut902 was found to have a relatively high significance (Table 5).

Table 6. Descriptive statistics for 14 microsatellite loci from sample of Korean otter.

Locus	N	Na	Size range	Ne	Ho	He	uHe	F	P-value
Lut435	70	4	116 ~ 132	2.222	0.543	0.550	0.554	0.013	0.5538
Lut453	70	6	122 ~ 132	2.312	0.429	0.567	0.572	0.245	0.0003***
Lut457	70	5	178 ~ 186	1.889	0.486	0.471	0.474	-0.032	0.1531
Lut604	70	6	118 ~ 130	4.487	0.771	0.777	0.783	0.007	0.0503
Lut615	70	7	239 ~ 257	4.075	0.743	0.755	0.760	0.016	0.3065
Lut701	70	17	189 ~ 253	5.475	0.757	0.817	0.823	0.074	0.2095
Lut715	70	12	162 ~ 202	5.932	0.643	0.831	0.837	0.227	0.0000***
Lut717	70	5	172 ~ 192	3.791	0.686	0.736	0.742	0.069	0.3201
Lut733	70	8	160 ~ 182	3.167	0.514	0.684	0.689	0.248	0.0000***
Lut782	70	7	161 ~ 189	3.845	0.657	0.740	0.745	0.112	0.1209
Lut818	70	9	155 ~ 181	4.354	0.557	0.770	0.776	0.277	0.0000***
Lut832	70	4	183 ~ 195	2.513	0.600	0.602	0.606	0.004	0.7024
Lut833	70	8	139 ~ 165	3.600	0.657	0.722	0.727	0.090	0.3663
Lut902	70	6	138 ~ 162	4.809	0.771	0.792	0.798	0.026	0.1643
Mean		7.429		3.748	0.630	0.701	0.706	0.098	

N: number of individual, Na: number of alleles, Ne: number of effective allele Ho: observed heterozygosity, He: expected heterozygosity, uHe: unbiased expected heterozygosity, ***: p-value < 0.001

Table 7. The result of null allele test for the nation wide, the north population, and the south population.

Locus	National wide (N=70)		North population* (N=28)		South population* (N=27)	
	Presence	Brookfield frequency	Presence	Brookfield frequency	Presence	Brookfield frequency
Lut435	No	0.0046	No	0.0349	No	0
Lut453	Yes	0.0886	No	0.0501	No	0.0522
Lut457	No	0	No	0.05	No	0
Lut604	No	0.0032	No	0	No	0.0197
Lut615	No	0.0067	No	0	No	0.0096
Lut701	No	0.0331	No	0.0287	No	0
Lut715	Yes	0.103	No	0.0505	Yes	0.1168
Lut717	No	0.0291	No	0.0404	No	0
Lut733	Yes	0.1009	No	0	No	0.0753
Lut782	No	0.0476	No	0.0083	No	0.0471
Lut818	Yes	0.1204	No	0.0603	No	0.0758
Lut832	No	0.0013	No	0.231	No	0
Lut833	No	0.0378	No	0.0497	No	0.0357
Lut902	No	0.0115	No	0	No	0

* North population: Han River population and East coast population, South population: South coast population.

The level of genetic diversity of Korean otter population was compared to those of European otter population (Table 8). Number of alleles and expected heterozygosity of Korean otter population were slightly lower than those of European population.

Table 8. Comparison of the degree of genetic diversity between Korean and European otters.

Locus	Korean population(n=70)				European population (n=102)**			
	Alleles		Ho	He	Alleles		Ho	He
	N	*MW*			*N*	*MW*		
Lut435	4	116-132	0.54	0.55	12	123-151	0.61*	0.83
Lut453	6	122-132	0.43*	0.57	9	113-139	0.69*	0.82
Lut604	6	118-130	0.77	0.78	9	120-140	0.43*	0.75
Lut615	7	239-257	0.74	0.76	11	240-268	0.63*	0.83
Lut701	17	189-253	0.76	0.82	8	192-212	0.58*	0.76
Lut715	12	162-202	0.64*	0.83	6	197-217	0.46*	0.64
Lut733	9	160-182	0.53*	0.69	8	160-184	0.57	0.69
Lut782	7	161-189	0.66	0.74	8	177-201	0.54	0.55
Lut818	9	155-181	0.56*	0.77	6	154-200	0.49*	0.76
Lut832	4	183-195	0.60	0.60	6	178-198	0.48*	0.69
Lut833	9	139-165	0.67	0.73	6	151-171	0.54*	0.78
Mean	7.57		0.63	0.71	8.09		0.55*	0.74

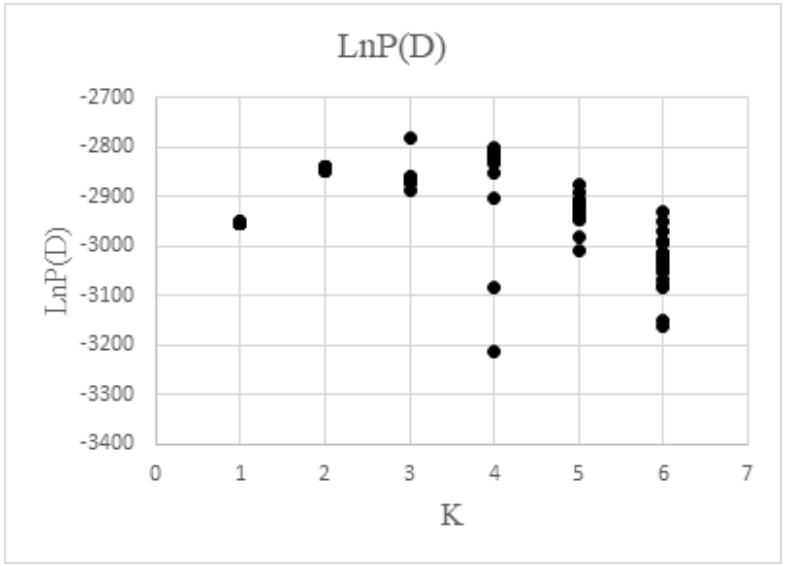
* Significant deviation from HWE at α=0.01

** E. Randi *et al.* 2002

N: number of allele, MW: range of allele size, Ho: observed heterozygosity, He: expected heterozygosity.

STRUCTURE was used for identifying substructure of Eurasian otter population in South Korea. For the analysis of population structure, the genotype data of 70 otter individuals are divided into six putative groups based on the watershed system. Six assumed otter subpopulations are composed of 15 individuals from Han River, 13 individuals from East coast, 4 individuals from Nakdong River, 3 individuals from Geum River, 8 individual from Seomjin River, and 27 individuals from South coast (Figure 3).

The method of Evanno *et al.* (2005) was used for estimating number of subpopulation (K) from posterior probability, LnP(d), based on Bayesian analysis (Figure 4). ΔK had the highest value when K is two, so this result presumed that two subpopulation were in South Korean otter population (Figure 4, 5). In the barplot, Han River and East coast populatons are grouped together, Seomjin River and South coast populations are assigned to the same cluster. Otter individuals from Nakdong River and Geum River are assigned to the middle group (Figure 6). When an additional STRUCTURE analysis with genotype data of only 10 microsatellite loci excluding loci with null allele detections, K was measured as 2 again. The bar plot results were also similar to those analyzed by 14 microsatellite marker data. These results indicate that there was little bias due to the null alleles.



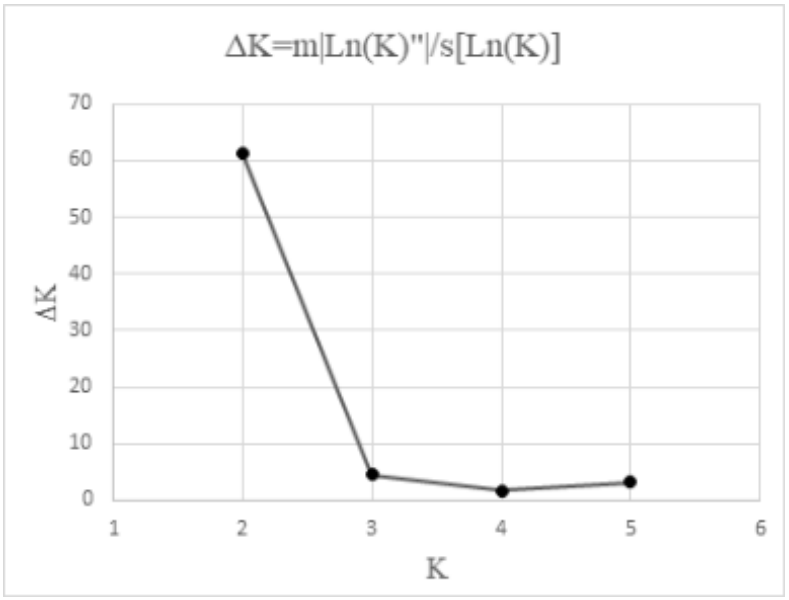
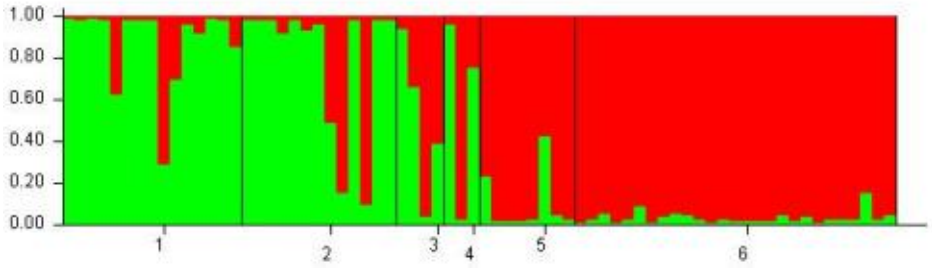


Figure 4. Estimation of the number (K) for river otter subpopulations in South Korea by using the method of Evanno *et al* (2005).

a.



b.

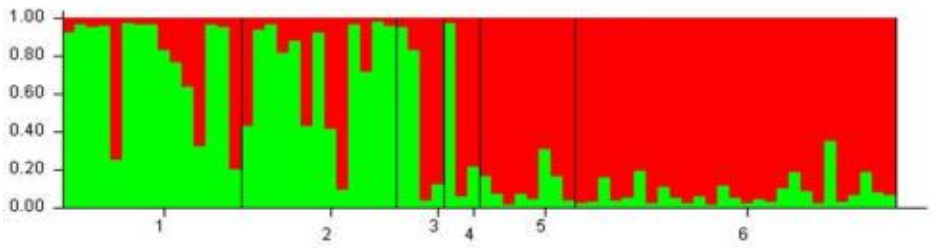


Figure 5. The barplot from STRUCTURE analysis, 1. Han River population, 2. East coast population, 3. Nakdong River population, 4. Geum population, 5. Seomjin River population, and 6. South coast population.

a: analysis by using 14 microsatellite markers

b: analysis by using 10 microsatellite markers excluding four loci with null allele detection.

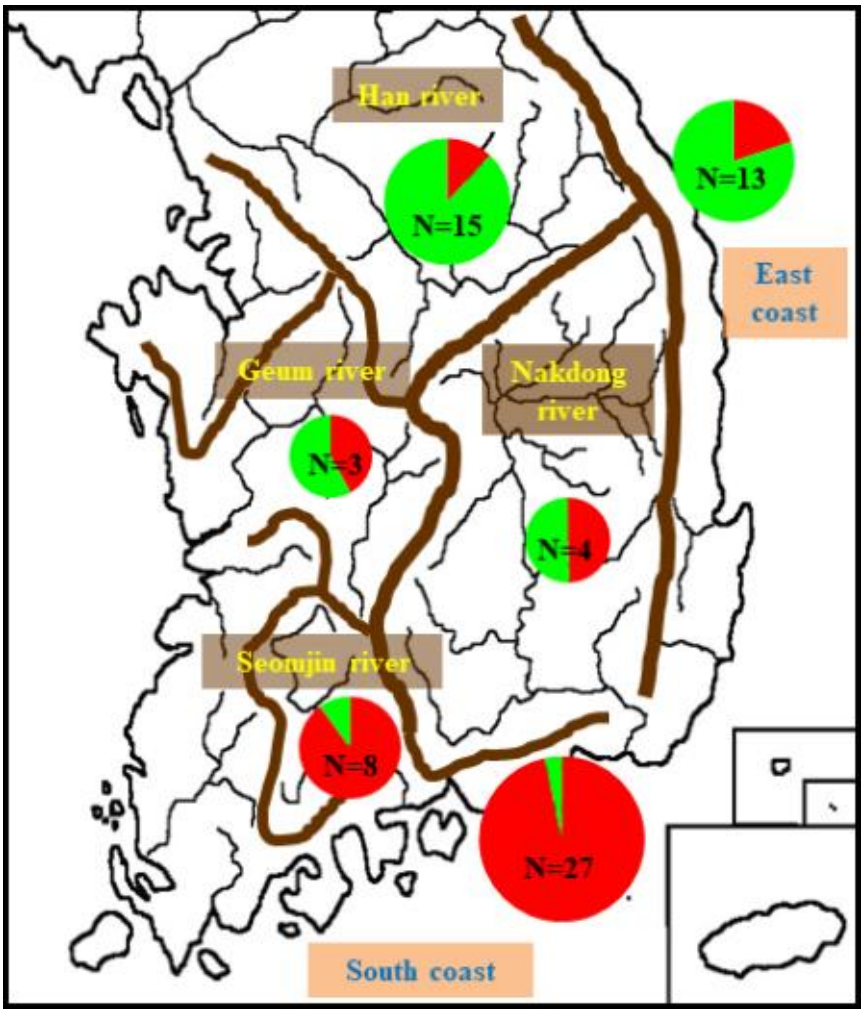


Figure 6. Map of study areas with sample number information. Pie charts show proportions of the STRUCTURE clusters.

The principal coordinates analysis (PCoA) also supported the result of Bayesian STRUCTURE analysis. Even though the explanatory power are relatively week (X-axis, 10.78%; Y-axis, 8.96%), the scatter diagram showed that the South coast population is distinct from Han River and East coast population (Figure 7).

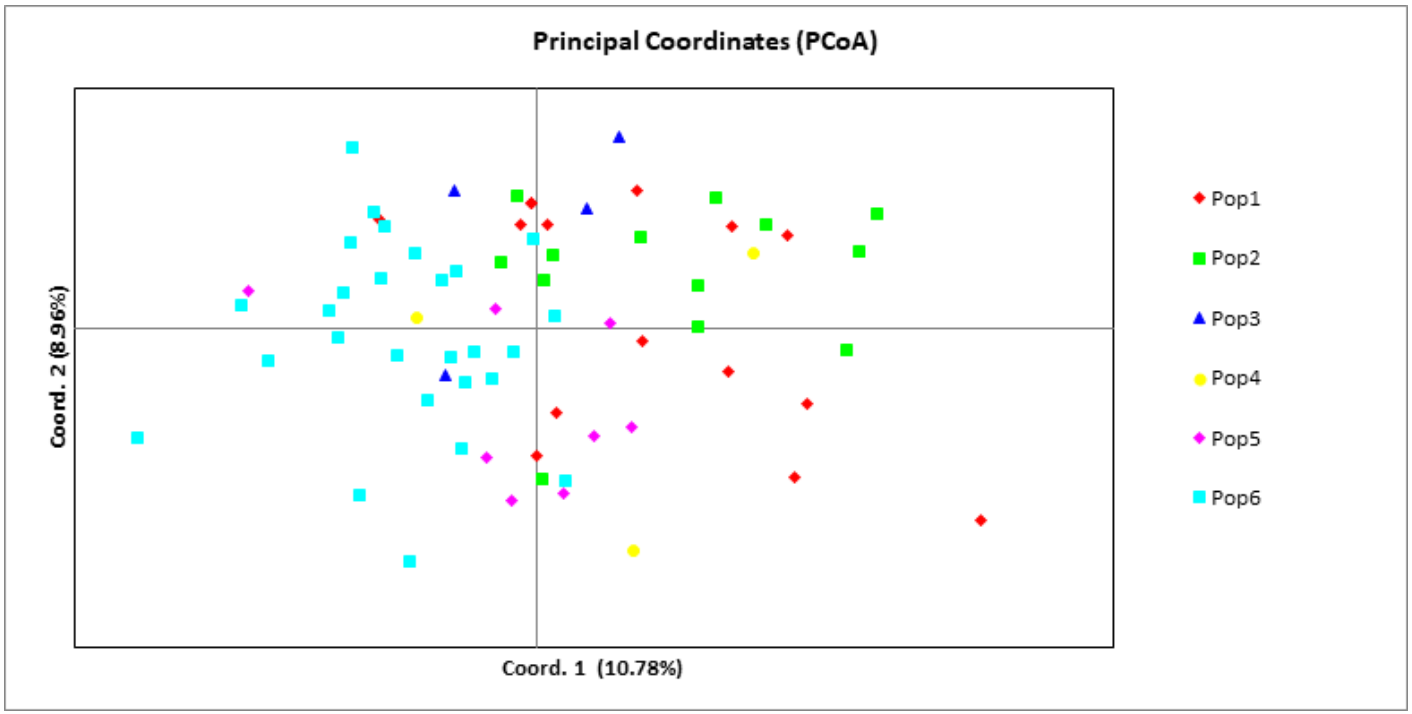


Figure 7. Scatter diagram from a principal Coordinates analysis. Pop1, Han River population; Pop2, East coast population; Pop3, Nakdong River population; Pop4, Geum River population; Pop5, Seomjin River population; Pop6, South coast population.

Based on the results of STRUCTURE analysis and PCoA, we reconstructed Korean otter population into 3 groups, Han River and East coast cluster (HEC), Nakdong River and Geum River cluster (NGC), and Seomjin River and South coast cluster (SSC), and performed F-statistics analysis. Weak population differentiation (F_{ST} = 0.0449) is detected between HEC and SSC, but the value of F_{ST} is significant by Bofferoni correction.

Table 9. Pairwises F_{ST} (below the diagonal) among three Eurasian otter clusters in South Korea and P-value (above the diagonal) obtained after 300 permutations.

	HEC	NGC	SSC
HEC	0	0.17667	0.0033*
NGC	0.0021	0	0.05
SSC	0.0449	0.0106	0

* Indicative of adjusted nominal level (5%) for multiple comparisons (0.0167).

For the analysis of isolation by distance (IBD), otter individuals collected from nearby locations were grouped together, and 58 individuals were grouped into 13 groups in this way. The correlation between genetic distance [$F_{ST}/(1-F_{ST})$] among 13 groups and geographic distances among 13 groups by using their GPS information was calculated through Mantel test for the IBD analysis using GenAlEX and Arlequin. The correlation coefficient (R^2) was 0.1232 and the P-value was 0.013. Although the correlation coefficient was significant in the case of the alpha value of 0.05, it could be said that a weak level of the IBD is confirmed because the correlation coefficient was not significant when the alpha value was 0.01 (Figure 8).

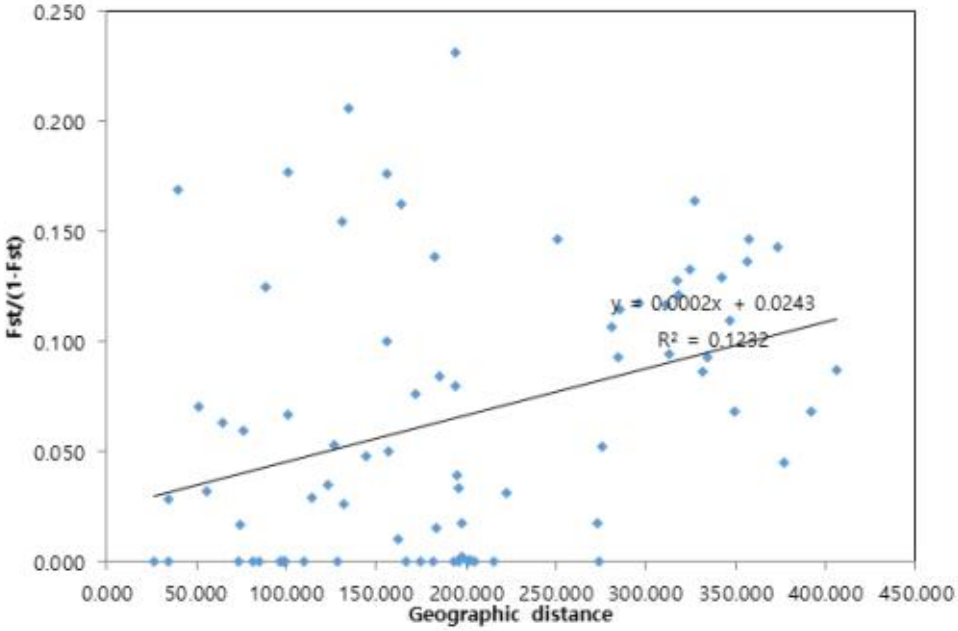


Figure 8. The result of isolation by distance (IBD) analysis with R^2=0.1232 and P-value=0.013.

Population bottleneck of Eurasian otter population in South Korea was identified based on sign test, standardized difference test, Wilcoxon rank test, mode shift distribution, and Garza and Williamson 'M'. The sign test, standardized difference test, and Wilcoxon rank test were performed under infinite allele model (IAM), Two phase model (TPM), and stepwise mutation model (SMM). When analyzing under TPM, proportion of SMM in TPM was 70%. The result of mode shift is normal L-shape distribution, and Garza-Williamson 'M' is 0.900. These results presume that the population bottleneck was not detected. However, the rest of three analysis indicate the probability of population bottleneck under infinite allele model. In the result of sign test under IAM, the number of loci with heterozygosity excess is larger than expected number of loci with heterozygosity excess under mutation-drift equilibrium significantly ($P<0.05$). Population bottleneck was identified with a strong significance from standardized difference test ($P < 0.01$) and Wilcoxon rank test ($P < 0.001$) under IAM. On the contrary to this result, population bottleneck was not detectable under TPM and SMM (Table 10).

Table 10. The results of population bottleneck analysis of Eurasian otter population in South Korea.

	IAM	TPM	SMM
Sign test			
- expected number of loci with heterozygosity	8.22	8.28	8.26
- number of loci with heterozygosity deficiency	2	5	10
- number of loci with heterozygosity excess	12	9	4
- Probability	0.03219	0.45945	0.02115
Standardized difference test - T2 (Probability)	2.885 (0.00196)	0.627 (0.26545)	-3.731 (0.00010)
Wilcoxon rank test - Probability of heterozygose excess	0.00031	0.27081	0.97528
Mode shift	Normal L-shaped distribution		
Garza and Williamson Test 'M'	0.900		

Discussion

Population structure

South Korea has undergone an industrialization process similar to that of Japan. After World War II and the Korean War, rapid industrialization deteriorated the natural environment and water quality, and otter habitats were destroyed. In addition, numerous numbers of otters were sacrificed for fur production during the war and industrialization process. As the result, otter populations in both South Korea and the neighboring Japan severely declined, and the Japanese population eventually became extinct, while Korean otter remained as an endangered species. Even though a small, reduced population of the South Korean otter persisted, it might have experienced a serious population decline and local population extinctions, and thus resulting in the shrinkage of distribution range and population fragmentation. Hong' research (2018) shows that there have been fragmentation of the population, although the reliability of the study was questioned because the study result heavily depended on the questionable data such as old records and reports (Figure 9). According to the study result by Hong (2018), Korean otter population was divided into two populations before 2000s (Figure 9). However, this result depend on occurrence data of otter trace, so there is possibility that the data may be poor to reveal the actual otter population structure due to the

following reasons. Firstly, Eurasian otter generally have wide habitat range (max. 22 km of male individual), and this species sometimes travel long distance over 80 km (Kruuk 2006). Therefore, there was a possibility that there had been an unidentified gene flow between two otter populations on the map of Figure 9. Secondly, there might have been undetected otter traces due to the limitation in otter monitoring methods in old days. In other words, there might have been unverified otter ranges on the map. Therefore, evidence of historical data on the map alone might be difficult to assure that otter populations have been fragmented.

However, the results of the study by Hong (2018) may be confirmed or supplemented by genetic data in this study. First of all, possibility of population substructure in Korean otter population were detected through the most basic analysis of population genetic data. Highly significant heterozygosity deficiency was detected on the four microsatellite loci, Lut453, Lut715, Lut733 and Lut818, (Table 6). This deficiency is likely due to the Wahlund effect. Wahlund effect occur when more than two different populations are treated as one. This heterozygosity deficiency was also confirmed in European otter data in Table 8. Since several otter subpopulations has been in Europe, most of loci had heterozygosity deficiency. Therefore, this deviation of observed heterozygosity from Hardy-Weinberg equilibrium (HWE) in

the four loci possibly means presence of more than two otter subpopulations in South Korea. In addition, these four loci may suggest the presence of null alleles (Table 5), but these signals of null allele is due to deviation from HWE. As shown in Table 7, absence of null alleles in North population and the result of the STRUCTURE analysis excluding the four loci with the null allele detection showed that the deviation of these HWEs could not be attributed to the null alleles. Moreover, detection of linkage disequilibrium between Lut782 and Lut902 (Table 5) also suggest the presence of population substructure.

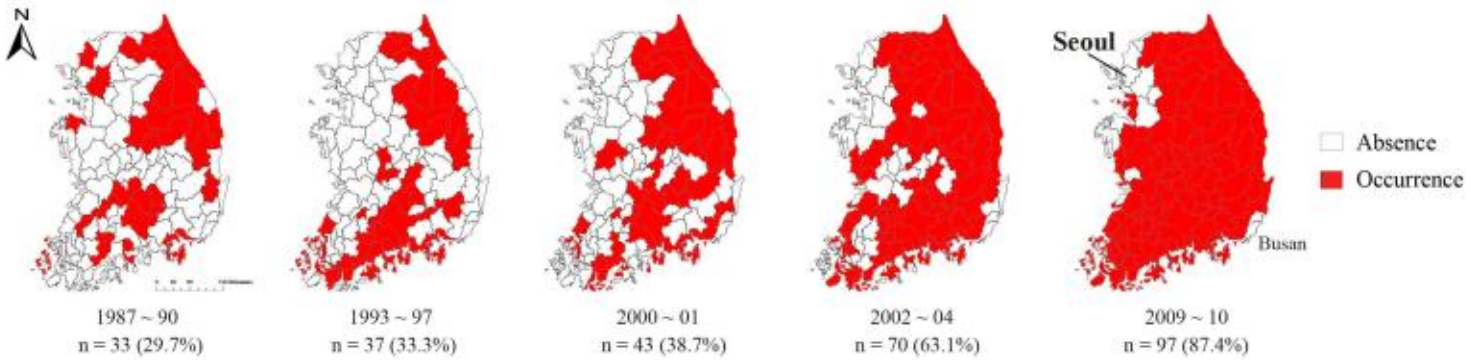


Figure 9. Changes in the distribution of otters (red) in 111 South Korean river basins from 1987 to 2010, based on field surveys in 1987 – 90, 1993 – 97, 2000 – 01, 2002 – 04, and 2009 – 10 (Hong 2018).

Secondly, the presence of two subpopulations of Eurasian otter in South Korea was confirmed by Bayesian analysis of STRUCTURE. The result of analysis by Evanno method estimated that K (number of subpopulations) value was 2, based on posterior probability data (Figure 4). This result support the possibility of Wahlund effect in the four microsatellite loci. Barplot of Figure 5 and pie charts on the map of Figure 6 showed the population structure of South Korean otter population. Eurasian otter individuals from Han River and East coast (HEC) were assigned to the same cluster, and those from Seomjin River and South coast (SSC) compose the same population. Nakdong River and Geum River (NGC) populations appear to be the intermediate form between two clusters above. As a result, it may be assumed that the Nakdong River and the Geum River serve as corridors connecting the two subpopulations of north and south. Moreover, considering that the Han River and East coast populations are bound together as a single population, it may be assumed that the mountain ranges are not genetic barrier for Eurasian otters in South Korea because the Tabaek mountain range is the highest one in South Korea.

However, two major clusters, HEC and SSC, were weakly differentiated based on result of F-statistics analysis (Table 8). This results imply that two subpopulations have recently been differentiated.

The low explanatory power in the result of principal coordinates analysis also supports this fact (Figure 7).

Cause of the population structure

Why current South Korean otter population is differentiated into two subpopulation despite the wide home range and dispersal length of the species? There could be three possible hypotheses explaining the causes for this population structure.

Firstly, the population structure may have been formed by simple effect of isolation by distance. The otter's habitat range is 13 to 21 km on a male basis (Han 1997), and there is a record about an individual traveled at one time up to 80 km (Kruuk 2006). However, this kind of long distance dispersal cases with reproduction success might be quite rare event, and the actual distance traveled may be limited by the check of other otter individuals already occupying the neighboring habitats. Therefore, limited dispersal ability of otter species may have contributed to the formation of population substructure even in relatively small area like southern part of the Korean peninsula. Even though the effect was not very strong, isolation by distance was confirmed in this study (Figure 8).

Secondly, it may be possible that the population structure was formed

by anthropogenic disturbance of otter habitat along the Nakdong River. During 1960s and 70s, the South Korean government extensively promoted rapid economic development and industrialization of South Korea, and one of the major geographic foci of the industrialization was Gyeonsangbuk-do province along the Nakdong River. Numorous industrial plants and cities such as Daegu, Gumi were constructed along the river, and many of them produced vast amount of contaminants polluting the river. The excessive pollution and development deteriorated water quality of the river and destroyed the habitat for otter population leading to the serious decline of local population along the Nakdong River (Jung *et al*. 2016; Kim and An 2015). Since the Nakdong River area population comprise the major part of middle group (Geum River and Nakdong River subpopulations) connecting the northern group (the Han River and East coast subpopulations) and southern group (Seomjin River and South coast subpopulations), local decline or extinction of Nakdong River subpopulation might have effectively fragmented otter habitat/corridor along the Nakdong River and blocked gene flow between the northern and southern groups for decades leading to the genetic differentiation of the two groups.

Finally, the population structure could have been formed by the fragmentation of the whole South Korean population due to the severe

bottleneck in the past. Population bottleneck was detected as a relatively strong significant level by the analysis using BOTTLENECK under infinite allele model (IAM). However, most of microsatellite loci have evolved under stepwise mutation model (SMM) or two phase model (TPM), so the results of BOTTLENECK analysis under IAM might be inappropriate. However, Hardy *et al.* (2005) reported that if mutation rate is significantly lower than migration rate or the bottleneck was within the 100 generations, there will be no contribution of stepwise mutation to genetic differentiation. In other word, the most recent population genetic events would follow the IAM than SMM. Therefore, detecting population bottleneck under IAM might mean that the population bottleneck event occurred most recently. Hajkova *et al.* (2007) study also detected recent population bottleneck under IAM model, and identified population structure among Czech and Slovak population of Eurasian otter. That study inferred that population fragmentation was due to dramatical population decline between 1970s and mid-1990. The statistically significant bottleneck signal was detected in Schleswig-Holstein otter population, which was recently recolonized from eastern part of Germany (Honnen *et al.* 2010). Therefore, the bottleneck test under IAM in this study might detect dramatically otter population declining due to rapidly industrialization and poaching before 1990s.

Taken together, it is most likely that the formation of current population structure of Korean otter is a composite result of these three causes rather than by a single factor. First, there was a bottleneck due to a rapid population decline in whole regions of South Korea, but especially severe decline in Nakdong River area in the past, resulting in population fragmentation. Since then, as the environment become improved, the otter habitat slowly recovered leading to the partial recovery of the otter populations. However, the population structure presumed to be still maintained partly due to the isolation by distance and the presence of anthropogenic barriers limiting the gene flow between the fragmented populations. In the future, detailed genetic monitoring of the otter population in Korean Peninsula including North Korea would be required to further clarify the cause of the population structure.

Genetic diversity

Comparing to the study of Randy *et al.* (2003), the number of allele and expected heterozygosity of Korean otter population are slightly lower than those of European ones (Table 8). The European otter population, like the Korean otter population, has undergone a drastic decline since the 20th century due to rapid industrialization and excessive poaching (Randy *et al.* 2003). Specifically, the otter populations in Central Europe have largely disappeared, but in some of

Western Europe, including the Iberian Peninsula, Ireland, and Scotland, Eastern Europe, including eastern Germany, and Northern Europe, the moderate size of otter population still remain (Randy *et al.* 2003). However, due to recent otter protection efforts and habitat improvements, some areas have undergone otter recolonization naturally, or some otter population have artificially reintroduced to extinct areas (Randy *et al.* 2003; Kruuk 2006; Geboes *et al.* 2016; Koelewijn *et al.* 2010). In this respect, the genetic diversity of the Korean otter (He=0.70) is similar to the genetic diversity of otters throughout Europe (He=0.74), and more specifically, slightly lower than the Ireland, which maintains a viable population size (He=0.77). Korean otter also has comparable genetic diversity with French (He=0.64, Geboes *et al.* 2016), Spanish (He=0.64, Mucci *et al.* 2008), and German otters (He=0.65, Mucci *et al.* 2008) that have fragmented populations. But genetic diversity of Korean otter is shown to be higher than that of the Danish otter population (He=0.38, Mucci *et al.* 2008) that is living in some areas due to the rapid population decline. Based on these results, it can be inferred that Korean otter populations have experienced a rapid population decline in the past, but maintained moderate genetic diversity.

Effectiveness of microsatellite marker for population genetics.

In this study, it was confirmed that microsatellite markers are very useful for population genetic studies of otters in Korea. Firstly, microsatellite marker detected possibility of population substructure using basic analysis of genotypic data. Secondly, this marker identified number of subpopulations and population structure using Bayesian analysis. Thirdly, the marker detected recent population bottleneck events and isolation by distance. Lastly, it estimated genetic diversity of Korean otter population and made the comparison with those of otter populations in other regions possible. Despite the development of methods for population genomics study using next generation sequencing (NGS), microsatellite markers seems to be still useful in population genetics and conservation genetic research.

Chapter 3: Individual Identification and Sex Determination of Eurasian Otters (*Lutra lutra*) in Daegu City Based on Genetic Analysis of Otter Spraints

Introduction

The Eurasian otter, *Lutra lutra*, is a species internationally protected by IUCN Redlist and CITES I because of population decline by overhunting, pollution, and habitat loss by industrialization. In South Korea, this species is also protected as an Endangered Species I and National Monument No.330 designated by South Korean government. The distribution, feeding habitat, habitat use, and heavy metal poisoning of Eurasian otter in South Korea have been reported (Han, 1997). However, studies that have effectively determined the population

size and genetic diversity of the animal have been not performed yet. Knowledge on the population size and genetic diversity of otters in local communities of South Korea is of great importance for designing effective strategies for conservation and management of the species by local governments. However, difficulties in observing and capturing these animals has hindered the direct estimation of population size, and thus only a rough estimation of otter populations has been made by investigating traces, such as footprints and scat, and habitat size. To overcome these difficulties, DNA recovered from samples collected by using non-invasive method, such as hair and scat, was used for individual otter identification using microsatellite loci analysis. These approaches have been successfully applied in studies of several wildlife species including the Eurasian otter (Creel *et al.* 2003; Kohn *et al.* 1999; Taberlet *et al.* 1999; Hung *et al.* 2004; Parsons *et al.* 2001).

Eurasian otters are scattered throughout South Korea, and only few individuals are found in any particular location. Recently, there has been a report on the appearance of otters in Daegu City, one of the cosmopolitan cities in South Korea well-known for its textile industry (Daegu Munhwa Broadcasting Corp., http://www.dgmbc.com/Special/050913.html). Otters had not been detected in the city until the early 2000s. Until recently, the Gumho River and Shincheon streams in Daegu City were severely polluted during the industrialization of the

city. However, the water quality of the river and streams in Daegu City has been improved with the efforts of the city government and citizens.

Since there had been no report on the appearance of otters in Daegu City until the early 2000s, it is assumed that otters currently found in this location are from regions outside of Daegu City. Therefore, it is important to understand the genetic status of otters recently found in the city as well as the size of the otter population in this area since these parameters can function as an indicator for genetic health of the otters and are fundamental components for successful settlement. This information can also be employed for designing effective management and conservation strategies for such protected animal in South Korea. In this study, we conducted microsatellite loci analysis using non-invasive methods to identify individuals and the gender of otter living in Daegu City. For this, we used DNA recovered from otter spraints collected in this area. Here we report the genetic diversity of the otters, minimum estimated size of the population, and relatedness among the individuals sampled.

Materials and Methods

Sampling and DNA extraction

Eighty-one otter spraints samples were collected from the Gumho River and Shincheon stream in Daegu City by Daegu Munhwa Broadcasting Corperation (MBC) teams as a part of documentary TV program about Eurasian otter living in the city. Most of these spraint samples were preserved in 100% EtOH and stored at -70℃. The fecal samples were selected by a visual survey and twenty fresh samples were subject to DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA).

PCR amplification and genotyping

Three sets of PCR primers were used. Firstly, to determine whether the genomic DNA was actually from an otter, we used otter-specific primers, LutcytF (5'-CCACAATCCTCAACAACTCGC-3') and LutcytR (5'-CTCCGTTTGGGTGTATGTATCG-3'), which were designed to amplify the partial cytochrome *b* sequence of otter. Polymerase chain reaction (PCR) amplification was performed with an initial denaturation for 5 min at 94℃, then 35 cycles of 94℃ for 30 sec, 50℃ for 30 sec, and 72℃ for 30 sec; and a final extension at 72℃ for 7 min. PCR was performed in a final volume of 10μL containing fecal DNA of otters (amounts of DNA were unknown) or 10 ng of DNA extracted from

otter tissue as a positive control, 0.8 mM dNTPs, 0.5 mM of each primer, and 1X PCR buffer with 1.5 mM $MgCl_2$, and 0.25 unit of i-Star Taq polymerase (Intron™, Seongnam, Gyeonggi-do, South Korea). PCR products were 2μL separated on a 1.2% agarose gel, and then the band intensities of the fecal DNA PCR products were compared with those of DNA from otter tissue.

Twelve microsatellite markers (Lut435, Lut453, Lut457, Lut604, Lut615, Lut701, Lut715, Lut717, Lut733, Lut782, Lut818, and Lut832) developed by Dallas and Piertney (1998) were used for PCR amplification for genotyping. We used the PCR conditions suggested by Dallas *et al.* (1999). Sizes of the alleles were determined using an ABI Prism 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) with the GENESCAN-500 ROX size standard, and GeneMapper v3.7 (Applied Biosystems, Foster City, CA, USA).

Lastly, in order to determine gender of each individual otter we used a specific P1-5EZ and ZFXYRb dye-labeled primer pair for amplifying the partial zinc finger protein coding gene. Amplified PCR fragments were digested with *Bsa*MI, and analyzed using the ABI 3730 XL DNA analyzer and GeneMapper with a slight modification of the method developed by Mucci and Randi (2007).

We repeated the PCR experiments for microsatellite genotyping and gender identification at least four times independently to reduce mistyping errors by allelic dropout and false alleles, following the multi-tube approach proposed by Taberlet *et al.* (1996).

Data analysis

Allelic diversity along with observed (Ho) and expected (He) heterozygosities were estimated using the GenAlEx v6.1 (Peakall and Smouse 2006). The program was further used to measure the probability of identity (PI) to estimate the average probability of two unrelated individuals (or siblings), drawn from a random mating population, having the same multilocus genotype by chance. PI is widely used as an indication of the statistical power of a specific set of loci markers (Peakall and Sydes 1996) and of the minimum number of loci required for reliable genetic tagging (Taberlet and Luikart 1999). The program also was used to calculate pairwise relatedness among fecal DNA genotypes. The program calculated the relatedness value (r) between individuals, which is twice the coefficient of kinship (F_{ij}) if two individuals are not inbred. The r values were calculated using formulas suggested by Queller and Goodnight (1989) and Lynch and Ritland (1999).

Results

Twelve out of 20 fecal samples produced DNA quantities and qualities similar to the DNA recovered from tissue (as positive control) according to the band intensities corresponding to the partial cytochrome *b* gene PCR products amplified by LutcytF/R. These twelve fecal DNA samples were therefore used for amplification using twelve microsatellite markers. Ten of the 12 fecal DNA samples were successfully genotyped by more than eight microsatellite markers. Since D10 was stored at -70℃ without 100% ethanol, this fecal sample was not successfully used for amplification using the microsatellite markers (Table 11). However, despite the problems with D10 due to different preservation conditions, indirect comparison for DNA quantities and qualities using partial cytochrome *b* gene amplification were useful for determining whether microsatellite analysis will proceed or not.

Eight distinct genotype distributions were detected in ten fecal samples. Fecal samples D67 and D74, and D78 and D79 showed the same genotypes, so they were regarded as identical to each other. New sample ID numbers, such as Das01 ~ Das08, were assigned to each distinct genotype. Except for Das07, the gender of the sources of

seven samples was successfully determined and found to include four males and three females (Table 11).

Table 11. Information of analyzed twelve otter fecal samples from Gumho River and Shincheon stream in Daegu City

Sample ID	Sampling location	Storage	No. of analyzed loci	Individual ID	Gender
D10	Gongsan dam	-70℃	0	-	-
D23	Nogok wetland	Ethanol, -70℃	12	Das01	Male
D38	Jangam bridge	Ethanol, -70℃	12	Das02	Male
D61	Jangam bridge	Ethanol, -70℃	4	-	-
D65	Nogok wetland	Ethanol, -70℃	8	Das03	Female
D67	Chimsan bridge	Ethanol, -70℃	12	Das04	Male
D68	Chimsan bridge	Ethanol, -70℃	11	Das05	Male
D73	Chimsan bridge	Ethanol, -70℃	12	Das06	Female
D74	Chimsan bridge	Ethanol, -70℃	12	Das04	Male
D77	Gongsan dam	Ethanol, -70℃	11	Das07	-
D78	Gongsan dam	Ethanol, -70℃	12	Das08	Female
D79	Gongsan dam	Ethanol, -70℃	12	Das08	Female

A total of 49 alleles was observed in the eight otter samples for the 12 polymorphic microsatellite markers. The number of alleles per locus in the eight samples ranged from 2 to 6 with a mean of 4.1. The level of genetic diversity was moderate with a mean H_O of 0.618 and a mean H_E of 0.602 (Table 12).

Table 12. Descriptive statistics for eight distinct individual otters

Locus	N	Na	Ho	He
Lut435	8	4	0.250	0.539
Lut453	8	3	0.875	0.570
Lut457	8	4	0.500	0.414
Lut604	7	3	0.857	0.571
Lut615	6	5	0.667	0.736
Lut701	7	4	0.714	0.643
Lut715	8	6	0.500	0.781
Lut717	7	5	0.714	0.745
Lut733	7	4	0.571	0.663
Lut782	8	3	0.625	0.602
Lut818	7	6	1.000	0.827
Lut832	7	2	0.143	0.133
Mean		4.1	0.618	0.602

N: number of analyzed individual, Na: number of alleles per locus, Ho: observed heterozygosity, He: expected heterozygosity

When seven microsatellite loci were combined as a set of marker loci, probability of identity (*PI*) was 1.5×10^{-5} for unrelated individuals and 8.1×10^{-3} for siblings. Therefore, more than seven loci were necessary to identify individuals at a *PI* level of 0.01 (Figure 10). Das04 and Das05 had the same genotype for the seven loci. For the remaining four loci, Das05 had homozygote genotypes sharing one of the alleles found in Das04. It is assumed that this result was due to low quality of the Das05 DNA since PCR successfully produced only one of five repetitive amplifications for these four loci. Moreover, Das04 and Das05 were from animals of the same gender, and the corresponding fecal samples were collected from locations that were only about 1 meter apart. Accordingly, we presumed that the two samples were from the same individual because there was a very low probability of identifying the same set of genotypes at more than seven loci ($PI < 0.01$) in two different individuals. Finally, we could identify at least seven individual otters from the fecal samples in this study.

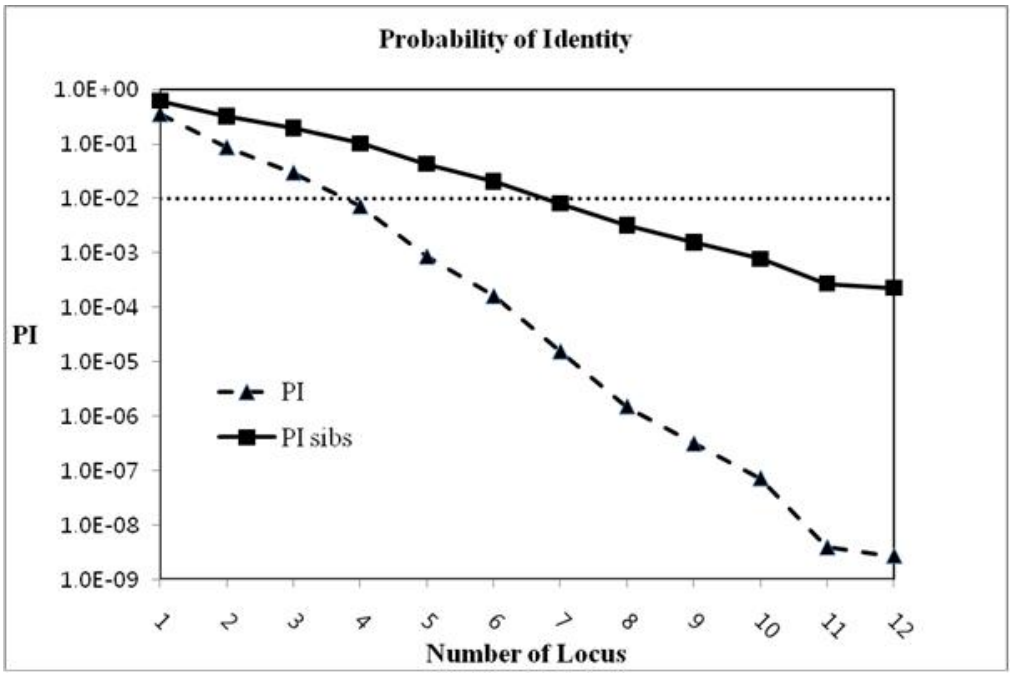


Figure 10. Relationship among the probability of identity (PI), PI among siblings, and the number of loci. The horizontal line indicates a PI of 0.01.

Pairwise relatedness (r) was estimated among individual otters (Table 13) based on formulas suggested by Lynch and Ritland (1999) and Queller and Goodnight (1989). The r value was calculated only when two genotypic data sets were comparable to each other at more than seven loci. In the Queller and Goodnight method, an r value close to 1 indicates an identical twin, a value close to 0.5 indicates a full sibling relationship (parents and offspring or brothers/sisters that share same parents), and an r value of 0.25 indicates a half sibling (brothers/sisters that share only one of parents). In the Lynch and Ritland method, r values around 0.5, 0.25, and 0.125 correspond to identical, full siblings, and half siblings, respectively. Among a total of 28 pairwise comparisons, three individual pairs showed kinship relation: r = 0.382 for Das01 and Das03, r = 0.703 for Das04 and Das05, and r = 0.403 for Das07 and Das08 (Table 13). The r value for Das04 and Das05, samples presumed to be identical, was higher than that for a full sibling. This result shows individuals whose fecal samples were collected from places located nearby each other were very likely full or half siblings. (Table 13, Figure 11).

Table 13. Relatedness analysis using the Queller and Goodnight method (below diagonal), and the Lynch and Ritland method (above diagonal)

	Das01	Das02	Das03	Das04	Das05	Das06	Das07	Das08
Das01(D23)		-0.016	0.129*	-0.139	-0.12	-0.088	-0.111	-0.132
Das02(D38)	-0.026		-0.255	-0.04	0.006	0.017	0.029	-0.119
Das03(D65)	0.382**	-0.580		-0.197	-0.176	-0.146	-0.095	-0.122
Das04(D67&74)	-0.450	0.156	-0.683		0.282**	-0.091	-0.185	-0.251
Das05(D68)	-0.367	0.128	-0.709	0.703**		-0.097	-0.166	-0.234
Das06(D73)	-0.332	0.096	-0.558	-0.057	-0.145		-0.038	-0.032
Das07(D77)	-0.296	0.111	-0.389	-0.427	-0.494	0.032		0.115*
Das08(D78&79)	-0.430	-0.119	-0.547	-0.462	-0.654	0.040	0.403**	

** Full sibling relationship, * Half sibling relationship

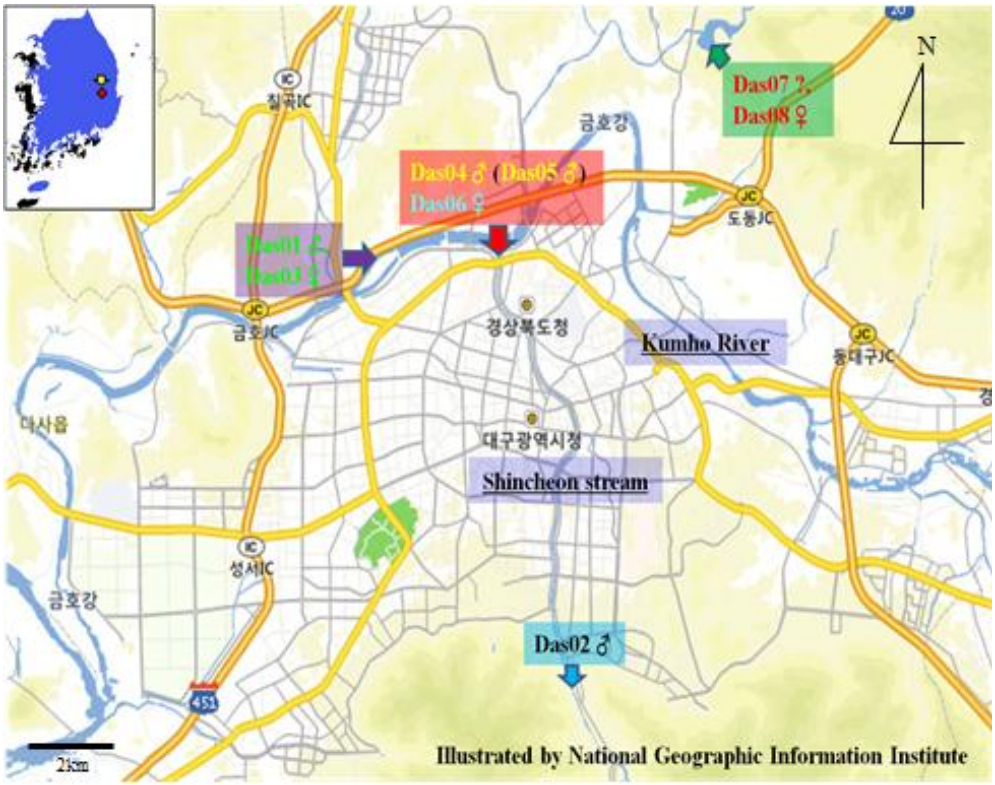


Figure 11. Sampling area locations according to each identified individual. Characters written in the same color indicate the individuals have a kinship closer than half sibling.

Discussion

A non-invasive analytic approach using otter spraint found in Daegu City have successfully provided information on the genetic diversity, minimum population size, and relationship among otters living in the area. It is estimated that more than seven Eurasian otters consisting of an almost equal number of males and females are living in Daegu City. The moderate level of genetic diversity of the otters in Daegu City (He = 0.60) is comparable with that of otters in Western Europe (He = 0.26 - 0.72 in Britain; He = 0.59 in France; He = 0.65 in Germany; He = 0.38 in Denmark; He =0.64 in Spain; He = 0.61 in Portugal), and Central Europe (He = 0.50 in the Czech Republic; He = 0.57 in Slovak; He = 0.57 in Austria; He = 0.64 in Hungary; He = 0.66 in Serbia-Montenegro) (Dallas *et al.* 2002; Mucci 2008). The moderate level of genetic diversity and the low degree of pairwise relatedness (89% unrelated and 11% kinship) among the otters in Daegu City may increase the probability of successful settlement of the animal in this area. It is desirous that at least seven otters consisting of several family groups inhabit Daegu City, an area in which there has been no report of the appearance of otters until the early 2000s. This means the local fresh water ecosystem as well as urban environmental condition in Daegu City have improved considerably. However, successful settlement of the otters will heavily rely on available food

sources in this urban habitat. Careful management and continuous surveillance by the local government to allow frequent movement of the otters between the Gumho River and Shincheon streams in Daegu City will help prevent inbreeding. Moreover, ecological and genetic affinity between otters in Daegu City and ones living outside of the city (i.e. the Nakdong River) will require successful settlement and continuous survival of the otters in Daegu City.

Since otters have been recently found in Daegu City, it is thought that otters have migrated from areas outside of Daegu City. Therefore, it is necessary to determine gene flow and genetic relationships between otter populations in Daegu city and those living outside of the city. Continuous monitoring of otter population sizes and genetic diversity in the local area will also be important. To do this, more comprehensive DNA analysis of fresh otter scat samples collected in Daegu City and its suburban areas is needed.

The non-invasive approach of using otter spraints has some advantages in genetic studies of this species. Firstly, the feces of otters can easily be found on rocks and sandy heaps in the middle and sides of streams and rivers. Secondly, otter spraint include several kinds of indigestible residue from prey such as skeletons, scales, shells, feathers, and hair, along with otter DNA in fresh samples.

Fecal DNA analysis has been applicable for population genetic studies with microsatellite markers, and could be a very powerful tool compared to analyzing DNA from blood or hair by collected directly from captured animals. On the other hand, the use of fecal DNA has some disadvantages in population genetic studies of otters because of a low credibility level of genotypic data derived from fecal DNA. A small quantity and poor quality of DNA extracted from otter sprain may result in genotyping errors, such as allelic dropout and false alleles, associated with PCR amplification. These errors can lead to false estimates of population size mainly by overestimation.

Even though the sample size used for this study was small, the results suggest that genotyping DNA from otter spraints using microsatellite markers is an alternative method for estimating population size and relatedness. However, this method has a risk of overestimating population size by genotyping error due to the low quality of fecal DNA. To reduce genotyping error, we carried out independent PCR amplifications at least four times for each sample and marker using a previously described multi-tube approach (Taberlet *et al.* 1996). The multi-tube approach allowed us to detect several allelic dropouts in the raw data of this study, and prevented overestimating the number of individuals. In addition, a high quantity of fresh otter

spraints would be required in order to obtain more accurate information on population size.

Conclusions

It was concluded that more than seven individual Eurasian otters inhabit the Gumho River and Shincheon stream, and among these three of the individual pairs have kinship relationships. Although otters are widely distributed in Daegu City with a moderate level of genetic diversity, continuous and close monitoring of this small otter population is necessary for successful settlement of otters in the local area. The genetic analysis of fecal samples performed in this study was confirmed to be very useful for ecological studies of endangered species such as Eurasian otters.

General Discussion

In this study, molecular phylogenetic status of Eurasian otter populations in Eastern Asia were identified and genetic diversity of South Korean otter population was evaluated. In addition, an ecological genetic study was conducted on otter population in Daegu City.

It was clear that the Japanese otter was phylogenetically separated from Eurasian otter populations including Korean, Chinese, and European populations. However, no clear evidence has been found to distinguish them as an independent species by analysis through genetic distance. According to the ML tree and the BI tree, it was confirmed that the Chinese otter (*L. l. chinensis*) and the European (*L. l. lutra*) otter were grouped into one group and the Korean otter (*L. l. lutra*) was formed into an outgroup. These results suggest that it is necessary to reevaluate the systematic and taxonomic relationships of East Asian otter populations. In addition, considering that two Eurasian otters with uncertain origins are bound together with a group of Chinese otters (*L. l. chinensis*), both individuals may also belong to the Chinese otter group (*L. l. chinensis*).

In the future, genetic studies on five subspecies (*L. l. barang*, *L. l. nair, L. l. monticular, L. l. aurobrunnea,* and *L. l. kutab*) in Southeast Asia and South Asia are necessary for reestablishing phylogenetic relationships in East Asian otter populations. A taxonomic study of

four subspecies(*L. l. nair, L. l. monticular, L. l. aurobrunnea,* and *L. l. kutab*), especially in India and its neighboring countries, will help to clarify the exact phylogenetic location of Japanese otters and to trace the evolutionary origins of the Eurasian otters. It is generally accepted that the genetic diversity of a regional population which served as the evolutionary origin of the species would be expected to be higher than peripheral populations. In this regard, South Asian region, including India, which has the highest level of subspecies diversity of Eurasian otters, is likely to be the origin of the Eurasian otter evolution. However, those subspecies have not been genetically studied yet. In the future, it would be important to analyze the genetic diversity of the 5 Eurasia otter subspecies in South Asian regions to clarify the relationships of Eurasian otter populations in their entire range.

Recently, several individuals of otters are rediscovered in Tsushima Island, which is suspected to be originated from South Korea (Nakanishi and Izawa 2019; Kyodo News 2017; Kyodo News 2018). The incidence could serve as a chance to raise public awareness on the important of otter conservation and management in Japan and East Asian region. It could be considered as an accidental or natural “reintroduction” of otters into the island, not an “introduction” or “invasion” because the rediscovered animals originated from the genetically most close population in Korean Peninsula, and belong to the same species according to the present study (Kyodo News 2018). Even if the rediscovered individual otters was migrated to Tsushima

Island with anthropogenic assistance, if the individuals are successful in survival and reproduction in the island, the “anecdotal reintroduction” event could serve as a strong motivation or impetus for public discussions on the development of potential, additional reintroduction project into the island using captive or rescued otter individuals of South Korea. It would be of utmost importance to systematically monitor the new inhabitants of the island including population, ecological and genetic monitoring. The monitoring will give us the necessary information on the developing strategy for conservation and management of the small, isolated population.

Based on the results of population genetic analysis on Korean otters, we confirmed genetic evidences supporting a group structure in Korean otter population. In the four microsatellite loci, the expected heterozygosity deficiency is significantly deviated from the HWE, suggesting the Wahlund effect. The results of STRUCTURE analysis based on Bayesian analysis also confirmed that there are two subpopulations. It is likely that the Korean otter has been greatly reduced in number as a result of rapid industrialization, habitat destruction, water pollution, and poaching for fur, as Europe and Japan have experienced in the past. In this respect, the population genetic structure identified in this study may be evidence of this past population decline. The BOTTLENECK analysis also supports the hypothesis that bottlenecks may have been present in recent years. Further studies are needed to clarify the exact cause of the population

structure. The additional studies may be carried out with extensive sampling covering whole range of current otter habitat in South Korea including fresh fecal sampling.

The persistence of population fragmentation by human activity can have a negative impact on the management of wild populations. Long-term, extensive genetic monitoring will help to confirm that genetic fragmentation due to past bottlenecks is being maintained or not. In order to determine the cause of the population structure, ecological monitoring should be performed by identifying characteristics of otter habitat. Based on these ecological monitoring results, an effective plan may be developed to manage otter habitats efficiently and improve unsuitable habitats. This effort will improve the connectivity between previously fragmented otter populations and will minimize the impact of past bottlenecks.

Population genomics study would be helpful to determine the cause of the population structure in South Korea. Population genomics has been actively researched recently, and will provide detailed information on the precise otter population structure, changes in historical population structure, and changes in historical effective population size.

As a result of ecological genetic studies on otters in Daegu area, significant information for conservation and management was obtained. Gender and individual identification was possible through genotype

analysis of fecal DNA. These genetic study identified that at least 7 otters live in the Gumho River and Shincheon in Daegu, and that the Daegu otter population maintains a moderate level of genetic diversity. Following the study, additional non-invasive genetic studies on the Daegu otter population was carried out in 2016 and 2019 (Park and Cho 2016, Park 2019-unpublished). These two studies identified more otter individuals and identified genetic diversity similar to the results of this study. These results indicate that otter population are stable in Daegu area. Whether the otter individuals appearing in inner city areas are isolated from external groups is an important issue. Therefore, it is necessary to improve connectivity with the external otter habitat, such as the establishment of ecological corridor, in addition to the efficient management of urban otter habitat for the protection of the otter population in Daegu. Non-invasive genetic monitoring of the entire population including both inner and outer subpopulations of the greater Daegu area will also help to evaluate the connectivity and to develop corridor planning.

Eurasia otters play an important role as an indicator species and keystone species in rivers and marine ecosystems. The presence of otters is an indication that the region is rich in biodiversity and high level of ecological and environmental health. Through this study, it was confirmed that these otters also live in a well controlled urban

environment. Therefore, for the stable management of Korean otter populations, otter inhabitation can be induced by improving habitat where otters are occasionally found, such as Han River in Seoul City area. If necessary, reintroduction of otters can be considered. These efforts will not only help to preserve otters, but they will also help to maintain aquatic species diversity and improve the health of the ecological environment.

In this study, I investigated the phylogenetic position of Japanese otters and raised the necessity of reestablishing phylogenetic relationships of East Asian otter populations. We also confirmed the group structure and genetic diversity of Korean otter groups. Finally, individual identification was performed by noninvasive genetic analysis of otter population in Daegu City area. However, we have identified several limitations in this study. Firstly, Due to the lack of research on other otter subspecies in South Asia and Southeast Asia, systematic taxonomic uncertainties still remain in Eurasian otter populations in Japan (historical) and East Asia. Secondly, the Korean otter population structure revealed in this study does not reflect the most current situation. Lastly, there was a possibility of sampling error due to insufficient number of fecal samples in Daegu otter studies. Therefore, it will be necessary to obtain genetic information of Eurasian otters in Southeast Asia and South Asia in the future, and to

continue population genetic monitoring for the Korean otter population. Consistent genetic monitoring of the Daegu otter population with more extensive fecal sampling will be also important.

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